FABRICATION AND IN-VITRO CHARACTERIZATION OF CHITOSAN DERIVATIVE AND STRONTIUM APATITE COMPOSITE SHEETS FOR PERIODONTAL TISSUE ENGINEERING

DISSERTATION

Submitted to The Tamil Nadu Dr. M.G.R Medical University

in partial fulfillment of the requirement for the degree of

MASTER OF DENTAL SURGERY

BRANCH II

PERIODONTOLOGY

2015 - 2018

CERTIFICATE

This is to certify that the dissertation entitled **"Fabrication and in-vitro characterization of chitosan derivative and strontium apatite composite sheets for periodontal tissue engineering"** is a bonafide record of the work done by **Dr. Shamna N.S,** under the guidance during her post graduate study period of 2015-2018. The dissertation is submitted to **The Tamil Nadu Dr. M.G.R Medical University, Chennai,** in partial fulfillment of the requirement of the degree of **Master of Dental Surgery in Periodontology, Branch II.** It has not been submitted (partial or full) for the award of any other degree or diploma.

Signature of the Guide

Dr. ARUN SADASIVAN
Professor,
Department of Periodontics
Sree Mookambika Institute of Dental Sciences, Kulasekharam,
Kanyakumari District,
Tamil Nadu-629161

Signature of the Co- Guide

Dr. ELIZABETH KOSHI
Head of the Department,
Department of Periodontics
Sree Mookambika Institute of Dental Sciences, Kulasekharam,
Kanyakumari District,
Tamil Nadu-629161

URKUND

Urkund Analysis Result

Analysed Document:	SHAMNA PLAGIARISM FILE.docx (D34329857)
Submitted:	1/4/2018 3:53:00 AM
Submitted By:	shamnalthafs8@gmail.com
Significance:	4 %

Sources included in the report:

Sathiyavimal S.docx (D34217846)
http://doras.dcu.ie/19371/
https://www.hindawi.com/journals/jnm/2014/194680/

Instances where selected sources appear:

14

CERTIFICATE II

This is to certify that this dissertation work titled **"Fabrication and in-vitro characterization of chitosan derivative and strontium apatite composite sheets for periodontal tissue engineering"** of the candidate **Dr. Shamna N.S.** with registration Number **241513302** for the award of **Master of Dental Surgery** in the branch of Periodontology, [Branch- II]. I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **4** percentage of plagiarism in the dissertation.

Guide & Supervisor sign with Seal.

Date:

Place:

SREE MOOKAMBIKA INSTITUTE OF DENTAL SCIENCES, KULASEKHARAM

ENDORSEMENT BY THE PRINCIPAL /HEAD OF THE INSTITUTION

This is to certify that the dissertation entitled **"Fabrication and in-vitro characterization of chitosan derivative and strontium apatite composite sheets for periodontal tissue engineering"** is a bonafide research work done by **Dr. Shamna N.S** under the guidance of **Dr. Arun Sadasivan, M.D.S**, Department of Periodontics, Sree Mookambika Institute of Dental Sciences, Kulasekharam.

Dr. Elizabeth Koshi, MDS
PRINCIPAL
Sree Mookambika Institute of Dental Sciences
Kulasekharam,
Kanyakumari District,
Tamil Nadu-629161

DECLARATION

I hereby declare that this dissertation **"Fabrication and in-vitro characterization of chitosan derivative and strontium apatite composite sheets for periodontal tissue engineering"** is a bonafide record of work undertaken by me and that this thesis or a part of it has not been presented earlier for the award of degree, diploma, fellowship, or similar title of recognition.

Dr. Shamna N.S,
MDS Student,
Department of Periodontics
Sree Mookambika Institute of Dental Sciences
Kulasekharam,
Kanyakumari District-629161

ACKNOWLEGEMENT

I thank my god for providing me with everything that I required in completing this work.

It is my proud privilege to release the feelings of my gratitude to several persons who helped me to conduct this work. Firstly, I would like to express my deep sense of profound gratitude to my honourable, esteemed guide to Professor. **Dr. Arun Sadasivan, MDS**, for the golden opportunity to do this work. His keen interest on me at every stage of my research, support, encouragement, useful suggestions and continuous guidance enabled me to complete my thesis successfully.

I owe a deep sense of gratitude to my honourable Principal, professor and head, department of periodontics, **Dr. Elizabeth Koshi, MDS**, for her prompt inspirations, timely suggestions with kindness and enthusiasm helped me to a very great extent made my work easy and proficient.

I would like to express my deep gratitude and sincere thanks to our chairman **Dr. C.K. Velayuthan Nair, MBBS, MS**, and our Director **Rema V. Nair MBBS, MD, DGO.,** and trustees **Dr. Vinu Gopinath MS, Mch**. and **Dr. R.V. Mookambika, MD, DM**., for their help and support to complete my study.

I am extremely thankful to **Dr. Shilpa Jayakumar MDS** and **Dr. Nikhil Das MDS,** for their co-ordination, co-operation and kind guidance.

I also express my deep sense of gratitude to **Dr. Manoj Komath, scientist F** (Biomedical wing SCTIMST), Poojappura, Trivandrum, **Mr. Adarsh,** Ph.D student of Biomedical wing, **Dr. Rajesh**, Head of Biogenix Reasearch Centre, Trivandrum, for provided all the necessary facilities of the institute, their technical help, advice, assistance in keeping my progress on schedule, without which my study would not have been possible.

I am thankful to **Dr. Sarath Babu Kurra** for providing me with this timely statistical analysis involved in this study.

I also thank all my friends, **Dr. Anju Unnikrishnan, Dr. Anina Mohan, Dr. Blessing Emmanual** and **Dr. Christina Ann Jose**, who have willingly helped me out with their abilities, more or less contributed to the preparation of this work report.

It is my privilege to thank my husband, **Mr. Althaf Syed Mohamed** for his constant encouragement, help and support throughout my study works.

I take this opportunity to express my greatest regards to my **Parents** for their co-operation, understanding and constant encouragement which were the sustaining factors in carrying out the work successfully.

CONTENTS

LIST OF ABBREVIATIONS

CD	-	Chitosan Derivative
CS	-	Chitosan
DMEM	-	Dulbecco's Modified Eagle Medium
DMSO	-	Dimethyl sulfoxide
e-PTFE	-	Expanded Poly Tetra Fluoro Ethylene
FTIR	-	Fourier Transformation Infrared Spectroscopy
FBS	-	Fetal Bovine Serum
GTR	-	Guided Tissue Regeneration
GTMAC	-	Glycidyl Trimethyl Ammonium Chloride
HA	-	Hydroxyapatite
Kv	-	Kilovolt
KDa	-	Kilo Dalton
µg	-	Microgram
mm	-	Millimeter
Mg	-	Milligram
Mpa	-	Megapascal
OD	-	Optical Density
PDL	-	Periodontal Ligament
PGA	-	Poly Glycolic Acid
PLA	-	Poly Lactic Acid
PCL	-	Poly Capro Lactone

PBS	-	Phosphate Buffered Saline
SEM	-	Scanning Electron Microscope
SA	-	Strontium Apatite
SBF	-	Simulated Body Fluid
SDH	-	Succinate Dehydrogenase
TE	-	Tissue Engineering
UTM	-	Universal Testing Machine
WSCH	-	Water Soluble Chitosan
XRD	-	X-ray diffraction
3D	-	Three Dimensional

LIST OF TABLES

LIST OF GRAPHS

LIST OF COLOR PLATES

LIST OF ANNEXURE

Abstract

Background

Periodontal regeneration is defined as regeneration of the tooth-supporting tissues including cementum, periodontal ligament and alveolar bone. A potential tissue-engineering (TE) approach to periodontal regeneration involves the incorporation of progenitor cells and instructive messages in a prefabricated three-dimensional construct and subsequent implantation of the construct into the defect site. The third generation guided tissue regeneration (GTR) membranes are based on the concept of TE. Biomaterials consist of biodegradable polymers and bioactive ceramics, are suitable for regenerative medicine. Chitosan, a biodegradable natural polymer and it possess excellent biological properties such as biocompatibility, antibacterial effect, and rapid healing capacity. It has several limitations including poor solubility under physiological conditions, to overcome these limitations, we focused on Chitosan derivative. Strontium, a trace element in the natural bones, can be substituted for calcium in hydroxyapatite, producing beneficial effects on bone, including stimulation of osteoblast differentiation, inhibition of osteoclast formation and bone resorption in-vitro. It also showed an excellent healing capacity. So we incorporating strontium in synthesized hydroxyapatite. Thus in this study an attempt is being made to fabricate new generation GTR membrane for periodontal tissue engineering application.

Aim of this study

The aim of this study was to fabricate GTR membrane of chitosan derivative and strontium apatite of varying concentration via freeze drying technique and comparing their in-vitro properties.

Materials and Methods

GTR membranes made of chitosan derivative and strontium apatite of varying concentration via freeze drying technique and comparing their in-vitro properties. Morphological Properties analysed using scanning electron microscope (SEM).The mechanical properties such as tensile strength, elongation break and tear strength of samples were determined by using universal testing machine. Average membrane thickness was measured using thickness gauge. Chemical analysis are analysed using Fourier transformation infrared (FTIR)Spectroscopy In-vitro degradation test of the scaffold were conducted by incubating the membrane in PBS at 37ºC for 1,5,9,13,17,21,26 and 29days. Invitro bioactivity test of the scaffold were conducted by incubating the membrane in SBF at 37ºC for 3 and 7 days. Cytotoxicity assessment are observed using L-929 mouse fibroblasts.

Result

All the fabricated scaffolds were highly porous and had interconnected pore structure. The mechanical properties of fabricated membranes observed. Among these, chitosan derivative- strontium apatite membrane(7.5 mg and 10 mg) possess increased thickness(mm)[0.50±0.02], chitosan derivative possess increased tensile strength and chitosan derivative- strontium apatite (7.5mg)exhibited highest tear strength(MPa)[0.65±0.05].Evaluating the chemical stability of the fabricated membrane by FTIR, results clearly indicate the strong bonding between chitosan derivative-strontium apatite composite membrane. For degradation analysis and in-vitro bioactivity composite containing strontium apatite experienced higher weight loss. In cell culture composite membrane showed positive response to mouse

fibroblasts L929 cell attachment, here chitosan derivative-strontium apatite(7.5mg) (85.69%)exhibited enhanced viability than chitosan derivative.

Conclusion

From the observation of the study it was concluded that chitosan derivative - strontium apatite composite membrane could be suitable for use as a GTR membrane. Further studies are needed for chitosan derivative -strontium apatite composite membrane for clinical use.

Key words: Chitosan derivative, GTR membrane, Periodontal regeneration, strontium apatite.

Introduction

Periodontitis is an inflammatory disease of the periodontal tissues, caused by microorganisms and calculus accumulation on the bacterial biofilm, leading to degradation of the connective tissues and alveolar bone and subsequent formation of soft tissue pockets around the root surface. The ultimate goal of periodontal therapy is the regeneration of lost supporting tissues, the apical proliferation and migration of the epithelium must be prevented**.** It results in healing by development of long junctional epithelium, which precludes regeneration and results in repair.[1]According to a position paper by the American Academy of Periodontology, periodontal regenerative procedures include soft tissue grafts, bone replacement grafts, root biomodifications, guided tissue regeneration (GTR), and combinations for osseous, furcation, and recession defects.[2]In the recent , attention has been focused more on regenerative and reconstructive therapies such as bone grafts, root conditioning, guided tissue regeneration, growth factor.

The term, guided tissue regeneration (GTR), was given by Gottlow in 1986. The theory of guided tissue regeneration is one that attempts to eliminate the apical proliferation of epithelium in support of other cells that will rise the chances of regeneration – bone and periodontal ligament (PDL).Guided tissue regeneration with barrier membranes has been proved to be effective in precluding epithelial and gingival connective tissue cells from migrating into the blood clot around the instrumented root surface. A physical barrier (membrane) is positioned to cover the region in which the regenerative process is to take place. In the space under the barrier, cells from periodontal ligament and bone colonize the blood clot, expressing their possibility for regeneration. The barrier membrane used for GTR can be commonly distributed into three generations of membranes. According to Gottlow's

Classification (1993),first generation membrane (Non-resorbable),second generation membrane(Resorbable) and third generation membrane (Resorbable material with growth factor).[3]

The first generation (non-absorbable) membrane, made from cellulose acetate (Millipore) was used as an occlusive membrane by Nyman et al.1982 .Later studies have exploited membranes of expanded polytetrafluoroethylene (e-PTFE) particularly designed for periodontal regeneration (Gore Tex Periodontal Material). Further non-resorbable barriers are titanium reinforced ePTFE, high-density-PTFE, or titanium mesh. Studies have revealed that titanium support of high-density PTFE membranes lead to superior regenerative capacity when associated to traditional expanded PTFE membranes normally due to the further mechanical support provided by the titanium frame against the compressive forces applied by the covering soft tissue .The main disadvantage is the required for second surgical procedure for the exclusion of the membrane.[4]

The second generation (resorbable) membrane was aimed to avoid the requirement for surgical procedure. There are two common classifications of bio-resorbable membranes: the natural and the synthetic membranes. Natural membranes are prepared of collagen or chitosan. Some complications, such as primary degradation, early loss of material, epithelial down growth along the material were informed after the use of collagen membranes. Synthetic materials made of polyesters e.g., poly glycolic acid (PGA) poly-caprolactone (PCL), poly lactic acid (PLA) and their copolymers were assessed. These materials are biocompatible, but by explanation they are not inert since some tissue reactions may be probable during

degradation which hypothetically influence wound healing and compromise regenerative result.[4]

As the theory of tissue engineering(TE) has established, third-generation membranes have developed, which not only act as barrier membranes but also as delivery devices to discharge agents such as antibiotics, growth factors, adhesion factors etc, at the wound site on a time or essential basis in order to form and direct natural wound healing in an enhanced manner.[4] They may be considered into the following sub divisions:- barrier membrane with growth factors, barrier membrane with antimicrobial activity and barrier membrane with bioactive materials.[5]

The TE technology to bone and periodontal regeneration associates three key essentials to enhance regeneration, they are Conductive scaffolds/Extracellular matrix, Signalling molecules and Stem/Progenitor cells.[6] This three dimensional extracellular architecture (scaffold) perform various functions, including the support of cell colonization, migration, growth and differentiation.[7] It should have highly interconnected pores to stimulate cell ingrowth and distribution throughout the matrix, as well as enabling the increase of neovascularization. The minimum pore size is considered to be ~100–150 mm. Several methods have been used to prepare such interconnected porous structures, as for instance foaming, fiber extrusion and bonding, 3D printing, phase separation, emulsion freeze-drying, porogen leaching, in situ pore forming, particle aggregation, electro spinning , supercritical fluids technology, and combinations of particles and cells.[8] Of these ,freeze drying most important technique and is works on the principle of sublimation. This process is divided into three steps for its better understanding; they are Freezing, Primary and secondary drying.[9] This technique has leads to form macroporous, interconnected

complex of polymer matrix that maintenances human osteoblast attachment and proliferation and timely onset of bone mineralization and resulting extracellular matrix deposition.[10]

Biomaterials consist of bioactive and bio resorbable substances which imitate the natural purpose of bone and stimulate in-vivo mechanisms of tissue regeneration. Such composite substances based on biodegradable polymers and bioactive ceramics, are appropriate for regenerative medicine.

Chitosan (poly-N-acetyl glucose aminoglycan), are the second most abundant natural carbohydrate biopolymer extracted from chitin. In recent times, importance in chitosan has increased due to its excellent biological properties such as biocompatibility, rapid healing capacity and antibacterial effect.[11] Further benefits of chitosan scaffolds for bone TE include the development of highly porous scaffolds with interconnected pores, osteoconductivity, and ability to improve bone development both in-vitro and in-vivo**.** It has several drawbacks to be utilized in biological system, including its poor solubility below physiological conditions and lesser bioactivity. Therefore, to overcome these drawbacks, researchers concentrated on the derivation of Chitosan.

In this present work, we trying to make it degradable as well as bioactive, so that modified chitosan will serve it function of guided tissue regeneration material according to normal physiological healing.

Bone is a complex of organic and inorganic substances such as, nano-crystallites, collagen fibrils and hydroxyapatite. Hydroxyapatite has ability to bind to both hard and soft connective tissues. It retains both osteoconductive and

osteogenetic properties. It consists of constituents such as phosphorus and calcium that prompt intracellular and extracellular reactions.[12] Strontium, a trace element in the natural bones. It can be substituted for calcium in hydroxyapatite, creating favourable properties on bone, including inhibition of osteoclast formation, stimulation of osteoblast differentiation and bone resorption invitro. It also showed an excellent healing that exhibit new bone, cementum and functionally oriented periodontal ligament.[13] So we are incorporating strontium in synthesized hydroxyapatite. Thus in our current study an attempt is being made to fabricate GTR membrane of chitosan derivative(CD) and strontium apatite(SA) of varying concentration and comparing their in-vitro properties.

Aims & Objectives

AIM OF THE STUDY

The aim of the study was to fabricate chitosan derivative and strontium apatite composite sheets by the method of freeze drying which can be used for periodontal tissue engineering and to compare their in-vitro characteristics.

OBJECTIVES OF THE STUDY

- To fabricate the GTR membranes by incorporating varying concentration of strontium apatite (SA-7.5 mg and 10 mg) into chitosan derivative polymer by the method of freeze drying.
- To characterize and compare the in-vitro morphological, mechanical and chemical properties of GTR membranes after their fabrication.
- To evaluate the degradation behaviour of each membrane by incubating the membrane in Phosphate buffered saline (PBS) for 1, 5, 9, 13, 17, 21, 26 and 29 days.
- To evaluate the in-vitro bioactivity of each membrane by incubating the membrane in simulated body fluid (SBF) for 3 and 7 days.
- To assess the cytotoxicity of fabricated composite sheets.

Review of literature

Guided tissue regeneration is a favourable application for repairing periodontal tissues, for using membranes by applying barrier membranes. According to a hypothesis formulated by Melcher[14], certain cell populations exist in the periodontium have the potential to generate new cementum, alveolar bone and periodontal ligament, they have the opportunity to colonize the periodontal wound. The hypothesis was experimentally documented and histologically proved by Karringet al.[15] The necessary for exclusion of epithelial and connective tissue cells of the gingiva from the wound led to development and use of GTR membranes. Bioaborbable membranes are made of a wide variety of materials, such as polylactic acid, polyurethane, collagen and chitosan.[16] The natural biopolymer chitosan is presently a subject of attention in tissue engineering.[17]

CHITOSAN

Chitosan are the second most rich natural bio polymers, extracted from chitin. Chitosan and its derivatives are commonly used for the preparation of the biodregradeble biomaterials. Chitosan of acetylation degree over 80% and average molecular wt nearby 350 kilo Dalton verified the foremost level of activity. It could be used as a base material for scaffold procedures and as modification outfits for presently used biomedical devices in improving tissue regeneration capability. It can develop the possibility of combinative approach of controlled drug release idea and tissue development in reconstructive therapy in the field of periodontics.[18]

The following major features of chitosan make this polymer beneficial for numerous applications:[19,20]

- It has a well-defined chemical structure.
- It can be chemically and enzymatically modified.

- It is physically and biologically functional.
- It is biodegradable and biocompatible.
- It can be treated into several products including flakes, fine powders, beads, membranes, sponges, cottons, fibers, and gels.
- It is non-toxic and safe to use.
- It binds to microbial and mammalian cells.
- It is, fungistatic, haemostatic and spermicidal agent.
- It is anti-inflammatory agent and antitumor.
- It accelerate bone regeneration.
- It is immune adjuvant and drug delivery agent.

Chitosan or chitosan-based composites have several unique properties for their use as a barrier membrane in GTR applications.[21] Another biomaterial of attention is Hydroxyapatite, which is a major component of human bone. It is used as bone substitute in the fields of dentistry and orthopaedics because of its good bioactivity, osteo conductivity and biocompatibility. But it is brittle and easy to fracture so it is hard to mould into a definite shape. In this study we incorporating strontium in hydroxyapatite in order to overcome limitations of hydroxyapatite.[22]

Composites containing hydroxyapatite and natural biopolymers ,chitosan are commonly used as biomaterials for TE. These materials combine the ultimate bioactive and natural polymer composited which imitates natural bone functions and stimulates in-vivo tissue regeneration mechanism. Therefore, a composite biomaterial of strontium apatite and chitosan derivative is estimated to show increased osteoconductivity, biocompactible, excellent healing capacity and

biodegradation together with adequate mechanical strength. So many fabrication technologies have been functionalized to process biodegradable and bioresorbable materials for TE into 3D polymeric scaffolds of high porosity and surface area. The conventional techniques for scaffold fabrication contain fiber bonding ,particulate leaching, membrane lamination, solvent casting, and melt molding, freeze-drying method etc. Among these we fabricated by using freeze drying method. The freeze-drying method for scaffold fabrication has give rise to macroporous, interconnected network of polymer matrix that maintenances human osteoblast attachment and proliferation ,early onset of bone mineralization and following extracellular matrix deposition.[22]

CHITOSAN-HYDROXIAPATITE COMPOSITE MEMBRANES

Yin et al in 2000,[23] prepared a composite composed of HA and a complex made by crosslinking of chitosan and gelatin with glutaraldehyde was established by sol-gel method. Chitosan sol was made by dissolving chitosan in aqueous acetic acid solution by stirring 24 h at room temperature. A glutaraldehyde solution was prepared by dissolving it in water. Hydroxyapatite powder was added in deionized distilled water and ultrasonicated until the HA powder was distributed in water. The slurry was held for 5h to let hydroxyapatite powder deposit. Then this deposited paste mixed with a chitosan solution below agitation .After gelatin was added and allow to dissolving of gelatin glutaraldehyde aqueous solution. Finally the resultant mixture with equally distributed HA powder and was transformed into a mold then air dried inorder to obtain composite plate. HA content is changed to handle composites with different hydroxyapatite percentage weight.

Zhao et al in **2002,**[24] studied the preparation of 3D hydroxyapatite-chitosan-gelatin complex composite scaffolds designed by phase separation system. A suspension was made by using hydroxyapatite and deionized water. After 0.5 hour stirring at room temperature the mixture was preserved ultrasonically until the HA powder was carefully dispersed in the deionized water. Then chitosan and acetic acid were added. After stirring overnight gelatin was added to this suspension held in 40℃ water bath. Then addition of a glutaraldehyde solution it was put into plastic petri dishes at 40℃ half an hour then quickly removed to a freezer at –40^{o}C to freeze the solvent and make solid-liquid phase separation. The mixture was retained at that temperature for 2 hours. And finally placed in a freeze-drier, freeze-dried for at least 30h obtaining in a foam which were cut into disks. Varying the HA content and the compositional variables of the new mixture allowed densities of the scaffold and control of the porosities .

Yamaguchi et al in **2003**[25], fabricated HA/ chitosan composites using a co precipitation method. A chitosan solution of 1.5 weight % made by mixing chitosan powder into distilled water enclosing 0.6 weight % of acetic acid and the chitosan solution was adding into 8.5 weight % of phosphoric acid solution. The attained chitosan/ phosphoric acid solution was slowly dropped into 3.7 weight % calcium hydroxide suspension stirring till pH 9±0.2. During this stage chitosan became insoluble and precipitated with small HA crystals forming chitosan/HA composite. The temperature was at 25℃ and releasing speed was 3.2 ml/min. The resultant mixture was aged for 24 hours upon constant stirring. The preparation of chitosan/hydroxyapatite composite using citric acid was as an aqueous solution of 50wt% citric acid was mixed into a mixture of chitosan -hydroxyapatite (20/80)

complex. Then the obtained mixture was then aged for 24h. Finally the precipitate was filtered and washed by distilled water. The outcome of citric acid on mechanical properties of the composite were examined.

Park J.S et al in 2003,[26] assessed the periodontal tissue regenerative effects of a chitosan/ collagen sponge applied to preclinical intrabony defects surgically formed in beagle dogs. In this study 4mm intrabony defects were surgically generated in the bilateral maxillary first and third, the mandibular second and fourth premolars. The surgical control group received a flap operation only, while the buffer control group was treated afterwards with a PBS/collagen sponge and the chitosan group was cured with a chitosan/collagen sponge . They were killed 8 weeks after the operation, and a comparative histological examination was achieved. The consequences demonstrate the favorable effect of the chitosan/collagen sponge on the intrabony defects of beagle dogs. The inhibited apical migration of epithelium and the rise in the amount of new bone and cementum advocate the effectiveness of chitosan in periodontal regeneration.

Ta Wei Chen et al in 2004,[27] prepared chitosan membranes by a thermal induced phase separation method, resulting treatment with nontoxic sodium hydroxide gelating and sodium triphosphate, sodium sulphite crosslinking agents. Effects of these reaction agents on chitosan membranes were assessed to survey the feasibility of using these membranes in GTR application. The primary results revealed chitosan membranes cross linked with sodium triphosphate and sodium sulphite had gel content of 53.5% and 52.2%r, respectively. The chitosan matrix gelated with sodium hydroxide dissolved totally during gel content measurement. Chitosan membrane treated with sodium triphosphate had lowest elastic modulus of

12.9 Mpa (Megapascal) as associated with other membranes treated with sodium sulphite(17.9Mpa) and sodium hydroxide (23.6Mpa). From SEM observations, sodium hydroxide gelated chitosan membrane had the smoothest surface morphology than others. Nevertheless, sodium triphosphate cross linked chitosan membrane had better cell adhesion and proliferation effects in cell culture test. All chitosan membranes degraded by about 23%~28% of initial weight after a 90-day in vitro shaking test. This study described that chitosan membranes were used as a barrier membrane for GTR.

K.H. Im et al in 2005, [28] fabricated organic and inorganic scaffolds by solid-liquid phase separation and following sublimation of solvent based on HA. The morphological and mechanical properties of the scaffolds were measured by varying content of HA. The bioactivity was assessed after scaffolds were occupied into Simulated body fluid during 7 days. This study reported that desirable pore structure, mechanical properties, and bioactivity of the hybrid scaffolds attained through controlling the ratio of HA and chitosan.

Zhang et alin 2005,[29] prepared and characterized nano-hydroxyapatite/ chitosan composite scaffolds. The nano-hydroxyapatite particles were made through a chemical method. They bound to the chitosan scaffolds very well. This method avoids the migration of nano-HA particles into surrounding tissues to a certain level. The morphologies, components, and biocompatibility of the composite scaffolds were examined. SEM, porosity measurement, thermogravimetric analysis, X-ray diffraction, X ray photoelectron spectroscopy, and FTIR were used to analyze the physical and chemical properties of the composite scaffolds. The biocompatibility was assessed .The composite scaffolds revealed better biocompatibility than pure

chitosan scaffolds. The effects advocate that the newly developed nano-HA/chitosan composite scaffolds may help as a good three-dimensional substrate for cell attachment and migration in bone TE.

Shyh Ming Kuo et al in 2006, [30] assessed chitosan as barrier membrane for GTR application. Three types of chitosan membranes ,each was gelated by sodium hydroxide, crosslinked by sodium phosphoric acid and sodium sulphite, were prepared to be evaluated by the following classifications: the mechanical strength to generate an effective space, the rapid rate to reach hydrolytic equilibrium in phosphate-buffered solution, and the ease of clinical manipulative operations. Therefore, standardized, trans osseous and critical sized skull defects were made in adult rats and the defective regions were enclosed with the specifically prepared chitosan membranes. After 4 weeks of recovering, varying degrees of bone healing were detected beneath the chitosan membranes in contrast to the control group. This study concluded that chitosan membranes were apparently suitable for GTR.

Ismail Zainol et al in 2008,[31]prepared and characterized water soluble chitosan(WSCH)/nano hydroxyapatite(nano-HA) composites using mixing technique.30% glycerol was added to produce flexible composite. The WSCH/nano-HA composites was prepared using casting and drying process. The composite was characterized using scanning electron microscope, X-ray diffraction and Fourier-Transformed Infrared spectroscopy to regulate the morphology of the composites and to finalize the presence of hydroxyapatite in the composites. Results showed that hydroxyapatite was distributed homogeneously in water soluble chitoson matrix. The mechanical properties of the composite were verified using Universal

Testing Machine. It was found that increasing nano-HA content in the composite will decrease the tensile strength.

Jung-A Shin et al in 2009,[32] assessed the effect of hydroxyapatite (HA)-chitosan (CS) membrane on bone regeneration in the rat calvarial defect. The consequence of this study is Surgical implantation of the HA - CS membrane resulted in improved local bone formation at both 2 and 8 weeks associated to the control group. The hydroxyapatite and chitosan membrane would be additional effective than the chitosan membrane in initial bone formation. They concluded the HA-CS sheath would be an effective biomaterial for periodontal bone regeneration.

TarunGarg et al in 2012,[33] Prepared Chitosan Scaffolds for TE using Freeze drying Technology. The morphology and size of the scaffold preparations were observed using scanning electron microscopy. They concluded that the viability of processing chitosan 3D scaffolds for TE applications using freeze drying technology. Freeze drying technology was used to precipitate chitosan from acetic acid solutions and used effectively as a drug delivery carrier, able to transfer active agents or biomolecules and growth factors.

Sun Mizo et al in 2012,[9] described fabrication of chitosan–hydroxyapatite macroporous interconnected structure along with the polymers, rheological properties of the material, cell proliferation and chemical bonding, alkaline phosphatase activity of the macroporous scaffold considered using freeze-drying method. SEM study exhibited macroporous architecture with interconnected pores. Elemental analysis evidently specified presence of calcium,

phosphorus and sodium along with oxygen and nitrogen in the scaffold. The FTIR examination indicated chemical bonding between both the polymers. The rheological testing was accomplished which exhibited no significant change. Human osteoblast seeded on CS–HA matrices showed viability for longer period of time and greater cellular proliferation .Rise in mineral deposition was examined using alkaline phosphatase assay which confirmed that CS–HA scaffold provided conducive environment for osteoblast proliferation and mineral deposition. The morphological and mechanical properties of the scaffold were found to be crucial for bone TE.

H. R. LE et al in 2012,[34] fabricated and evaluated the mechanical properties of chitosan composite membrane containing hydroxyapatite particles. The properties of hydroxyapatite content on the microstructure and mechanical properties of composites were observed. It was found that the Young's Modulus of the composites declines with hydroxyapatite content while the failure strength and strain rise with the hydroxyapatite content.

Huang Yet al in 2013,[35] prepared CS and strontium-substituted HA films on titanium. FTIR features by electrochemical deposition method containing strontium, Phosphate ,Calcium and Chitosan. The prepared coatings were observed by scanning electron microscop, energy-dispersive X-ray spectroscopy, FTIR and XRD investigations. The results specify that the CS/Strontium HA coatings ,morphology of flake-like rather than the needle-like crystal .The FTIR assessment shows that the typical vibration absorption peaks of chitosan emerged, SBF immersion test showed that the CS/Strontium HA coatings had encouraged carbonate-apatite development, demonstrating that the composite coating keeps good biocompatibility. In the

electrochemical corrosion analysis, that the CHI/Strontium HA coatings exhibited stronger corrosion resistance than pure Titanium.

P.A. Norowski et al in 2012,[36] in his study, electrospun chitosan membranes, cross-linked with 5 mM or 10 Mm geinipin, a natural crosslinker extracted from the gardenia plant, were assessed for suture pullout strength, crystallinity, and cytocompatibility. Ultimate suture pullout strength was considerably lower (51–67%) than that of commercially available collagen membranes. Crystallinity of the electrospun chitosan membranes decreased upon crosslinking by 14–17%. The molecular weight of the chitosan polymer was reduced by 75% during the electrospinning process. Uncross linked and genipin-cross linked chitosan membranes were cytocompatible and maintained fibroblast cell proliferation for 9 days. Uncross linked and genipin- crosslinked membranes did not stimulate monocytes to produce nitric oxide in vitro in the absence of lipopolysaccharide. In conclusion, chitosan membranes inhibited lipopolysaccharide- induced Nitric oxide production by 59–67% as compared to tissue culture plastic and collagen membrane. Advances are necessary in the tear strength of electrospun chitosan membranes for clinical application.

Kimberly T et al in 2013,[37] assessed the feasibility of the hydroxyapatite–chitosan–gelatin composite as a barrier GTR membrane by examining the interfaces of the hydroxyapatite–chitosan–gelatin membrane with serum proteins and their following effects on Human mesenchymal stem or stromal cells properties. The results reveal hydroxyapatite–chitosan–gelatin prominent capacity to improve with extracellular matrix proteins, forming instructive microenvironments that encourage

Human mesenchymal stem or stromal cells proliferation and the advance of osteogenic differentiation.

Tao Sun et al in 2014,[38] fabricated chitosan and HA/chitosan scaffolds with desired pore sizes and porosity using thermally induced phase separation technique. The scaffolds were characterized using various methods. The invitro degradation and the response of fibroblast cells on porous chitosan-based scaffolds were also assessed. The scaffolds were highly porous and had interconnected pore structures. The combined HA nanoparticles were well mixed and physically existed with chitosan in composite scaffold structures. The addition of 10% HA nanoparticles to chitosan improved the compressive mechanical properties of composite scaffold related to pure chitosan scaffold. In vitro degradation effects in phosphate buffered saline (PBS) shown slower uptake properties of composite scaffolds. Moreover, the scaffolds showed positive reaction to mouse fibroblast L929 cells attachment. Generally, the findings advocate that HA/chitosan composite scaffolds could be suitable for TE applications.

L. Pighinelli, M. Kucharskain 2014,[39] fabricated preparation of certain forms of the early chitosan like microcrystalline chitosan, physico-chemical characterization, elaboration of the method for preparation composites with microcrystalline chitosan and hydroxyapatite. To increase the suitability of chitosan and its derivatives for bone TE, the composites of microcrystalline chitosan and hydroxyapatite could be applied. They determined that the sponge preparations with hydroxyapatite and microcrystalline chitosan formed a 3-D structure which can be used in future as a base

for scaffolds . The HA aggregates well in the polymer matrix of microcrystalline chitosan showing a standardised construction and distribution in the polymer matrix.

Bavariya AJ et al in 2014,[40] assessed biocompatibility and degradation of chitosan nanofiber membranes, with and without genipin crosslinking as related with a commercial collagen membrane in rat model which were inserted subcutaneously in the backs of 30 rats. The membranes were inspected histologically at 2, 4, 8, 12, 16, and 20 weeks. Sections were observed and graded by a blinded pathologist using a 4-point scoring system to decide the tissue reaction to the membranes and to detect membrane degradation. There was no statistically significant difference in histological scores between chitosan and collagen membranes at different time points. Absence or minimal inflammation was detected in 57–74% of the membranes across all groups. Most collagen membranes gone by resorption at 12–16 weeks. The general tissue reaction was related to that of control commercial collagen membrane. Still, the chitosan membranes exhibited slower degradation rates than collagen membranes.

Diana Marcela Escobar-Sierra et al in 2015,[41] fabricated chitosan/ hydroxyapatite scaffolds, using various ratios and two different techniques. The powder hydroxyapatite (commercial) and in situ hydroxyapatite, and then compare their properties. The morphology, chemical composition and mechanical properties were assessed by Scanning Electron Microscopy ,X-ray diffraction and compression tests. The scaffolds showed an interconnected porous structure. The scaffolds with chitosan and hydroxyapatite developed by in situ protocol, have improved applications inTE, because they have a better morphology and allow the cell growth. **Nitin Sahai et al in 2015,**[42] fabricated and characterized scaffolds (PCL, HA,

PGA& Chitosan)for tissue engineering applications. The actual mechanical strength/properties like stress and strain fall with the rise of the porosity for all three scaffolding biomaterials (HA, PCL, PGA). Chitosan scaffold displays same type deviation in there mechanical properties as with the rise in its porosity its mechanical properties declines but the mechanical strength of the Chitosan is very low. Lyophilization and Freeze drying are the techniques are used to create the porous chitosan tissue scaffold through which the size of porous tissue scaffold is controlled which will be supportive in fabrication of correct mechanical strength tissue scaffold.

Tu Ying et al in 2017,[43] fabricated an asymmetric nano-hydroxyapatite/ chitosan (n-HA/CS) composite GBR membrane was by means of solution-blending and solvent-evaporating in vacuum. The membranes were analysed using SEM, XPS and contact angle. It was create that the composite membrane displayed an asymmetric structure, in which the upper surface was CS and the under surface was a complex of n-HA and CS, and some relations between n-HA and CS were also confirmed to exist. The contact angle testing exhibited that the under surface was more hydrophilic than the upper surface. The in- vivo experiments revealed that the asymmetric composite membrane had the capability to make osteoblasts mineralize and support loose bone calcified, and then speed up the bone regeneration. Compared with CS membrane, the asymmetric composite membrane shows a better bone regeneration ability and is proper for GBR membrane.

Sang Min Park et al in 2017,[44] prepared composite membrane by blending acetylated chitosan with carbonated nano-size hydroxyapatite for use as a GTR barrier. The carbonate group of CHAP was a partial replacement of the hydroxyl

group and/or phosphate group of hydroxyapatite by sintering with carbon dioxide. Chitosan/CHAP complexes were acetylated with acetic anhydride to form the ACS/CHAP composites. The compositions and properties of the composites were confirmed by FTIR, inductively coupled plasma mass spectrometry, zeta potential analysis, X-ray diffraction analysis, UTM analysis, SEM, MTT assay, etc. The surface energies of the composites were improved by carbonation and acetylation. The acetylation of chitosan better the lysozyme degradation of the composite. The carbonation of hydroxyapatite significantly improved the viability of osteoblast-like cell on the composite. The high viability and intact phenotype of cell occurred on composite with ACS/CHAP ratio of 50/50, which had adequate elastic modulus for a GBR barrier.

Materials & Methods

The study protocol was approved by Institutional Research Committee(IRC) with Ref no.11/07/2016 and Institutional Human Ethics Committee(IHEC) protocol no 13/2016, Sree Mookambika Institute Of Dental Sciences Kulasekharam, Kanyakumari Dist, Tamil Nadu. Laboratory facilities for this study was provided by Bioceramics division, Biomedical technology wing of Sree Chitra Thirunal Institute of Medical Science and Technology, Trivandrum, India for a period of 6 months.

MATERIALS

- Group I - Chitosan derivative
- Group II - Chitosan-Strontium Apatite Composite (7.5 mg)
- Group III - Chitosan-Strontium Apatite Composite (10 mg)

For membrane fabrication

- Chitosan [India Products, India] [CP-1]
- Strontium apatite (wet precipitate of calcium and phosphate salt) [Bioceramics division, Sree Chitra Thirunal Institute of Medical Science and Technology, Trivandrum][CP-2]
- Glycidyltrimethyl ammonium chloride(GTMAC)[Sigma Aldrich Pvt Ltd., USA]
- Acetic acid(CH3COOH) [Sigma Aldrich Pvt Ltd., USA][CP-3]
- Methanol (Merck EMPARTA® Pvt Ltd USA)[CP-4].
- Conical flask containing magnetic bead (Bioceramics division, Sree Chitra Thirunal Institute of Medical Science and Technology, Trivandrum)[CP-5]
- Dialysis membrane (spectra/Por molecular porous membrane)[CP-6]
- Automatic stirrer (IKA C-MAG HS 7 digital Pvt Ltd, USA)[CP-7]
- Electronic weighing machine (Scaletec Mechanotronics Pvt Ltd, India)[CP-8].
- Freeze dryer (Benchtop SLC Pvt. Ltd. UK)[CP-9].

For analysing morphology

- Scanning electron microscope (Hitachi –model-s-2400, JEOL, JSM-6390, model 7582, Japan)[CP-10].
- Image J software.

For analysing mechanical strength

- Thickness Guage [CP-11].
- Universal testing machine (instron3345 single column, UK: software-bluehill3)[CP-12].

For chemical analysis (presence of functional groups)

- FTIR Spectrometer[CP-13]

For analyzing in-vitro degradation and in-vitro bioactivity

- PBS with PH of 7.4 [Bioceramics division, Sree Chitra Thirunal Institute of Medical Science and Technology, Trivandrum].
- SBF [Bioceramics division, Sree Chitra Thirunal Institute of Medical Science and Technology, Trivandrum].

For cytotoxicity assessment

- UV irradiator for sterilization of samples (Biogenix Research Center, Trivandrum, India).
- Mouse fibroblast L929 cells (National Centre for Cell Science, Pune, India).
- Dulbecos Modified Eagles Medium (Biogenix Research Center, Trivandrum, India).
- Phase Contrast Microscope [CP-14](Olympus CKX41) for MTT assay observation provided by Biogenix Research Center ,Trivandrum, India.

- Imaging software Optika vision-pro.
- Succinate dehydrogenase enzyme (SDH) [Biogenix Research Center, Trivandrum, India].
- PBS with PH of 7.4 [Bioceramics division, Sree Chitra Thirunal Institute of Medical Science and Technology, Trivandrum].

METHOD OF GTR MEMBRANE FABRICATION

The method employed in this study have been divided into following steps,

- Preparation of modified chitosan
- Preparation of strontium apatite containing chitosan membrane.

(1)Preparation of modified chitosan

2% chitosan solution was prepared by dissolving 1 gm purified chitosan in 50 ml, 2% acetic acid stirred overnight .When it was completely dissolved in acetic acid heated to 80℃ and 0.75 mL glycidyltrimethyl ammonium chloride was added, stirred at 80℃ for 7 hours in a conical flask containing magnetic bead. After 7hrs it was allowed to reach at room temperature and then precipitated in 150 ml acetone. The precipitate was filtered and washed with methanol. The precipitate again washed two times with methanol, filtered and dialysed for 24 hrs[CP-15].

(2) Preparation of the chitosan derivative/strontium apatite composite

Composite scaffold were synthesized with chitosan derivative and strontium apatite of varying concentrations (SA-7.5 mg and 10 mg). Strontium apatite powder was dispersed in deionized water for 2 hour. Subsequently suspention was added drop by drop to the chitosan solution(7.5 mg and 10 mg of strontium apatite in chitosan solution of 5 ml in each beaker).Next, the chitosan derivative/strontium

apatite suspension vigorously mixed using a magnetic stirrer for 2 hours to obtain homogenous mixture and it was transferred to containers, frozen and lyophilized for 24 hours[CP-16].

FOR ANALYSING MORPHOLOGY

The morphology of the composite sheets was observed by Scanning electron microscopy(Hitachi –model-s-2400,JEOL,JSM-6390 ,model 7582,Japan).The samples were dried and was coated with gold and analysed under scanning electron microscope (20 kv).

MEMBRANE THICKNESS

Average membrane thickness was measured using thickness guage[CP-11].The average thickness of the membrane at five random position was adopted as the mean thickness of the membrane.

FOR ANALYSING MECHANICAL PROPERTIES

The mechanical properties such as tensile strength and elongation break of samples were determined by using universal testing machine (Instron 3345,single column, UK: software –blue hill 3).A load cell of 100 N was hammered vertically at the speed of 5 mm/min on a membrane sample of 1x6cm.The tensile strength measurements were charted up to the point where they were broken[CP-17].

SUTURE PULLOUT STRENGTH

Suture pull out tests were analysed to determine the tear strength of the sheets. Membrane specimens were prepared to be 10 mm wide and about 40 mm long. A single suture was made 5 mm from the top edge and 5 mm from each side. The suture was a 70 cm general closure monofilament silk with taper ct-1 needle and

1 (4.0 metric) gauge. The suture was left un-knotted but was affixed to the upper claw of the Instron TM model 3345, mechanical test frame .Suture pull out testing of dry specimens was carried out with a 50 N load cell and an extension rate of 1 mm/min.[CP-18].

CHEMICAL CHARACTERISATION OF SCAFFOLDS

The presence of strontium apatite on composite sheets was analysed using FTIR.

IN-VITRO DEGRADATION TEST

In-vitro degradation test of the scaffold were conducted by incubating the membrane in PBS at 37ºC for 1,5,9,13,17,21,26 and 29days.For weight loss studies circular samples with 20mm diameter where incubated in a closed bottle containing 30ml phosphate buffered saline(PBS) having pH of 7.4 at 37ºC. The initial weight of the membrane before incubating in the PBS was measured and the membranes retrieved after 29 days. The retrieved membranes were washed with deionized water, dried in vaccum oven and weighed until constant weight is attained. The percentage weight loss was estimated using the equation,

$$\text{Weight loss } (\%) = (Wi - Wf)/Wi \times 100$$

IN-VITRO BIOACTIVITY TEST

Invitro bioactivity test of the scaffold were conducted by incubating the membrane in SBF at 37ºC for 3 and 7 days. For analysing weight difference circular samples with 20mm diameter where incubated in a closed bottle containing 30ml SBF at 37ºC. The initial weight of the membrane before incubating in the SBF was measured for 3 and 7 days and the membranes retrieved after 3 and 7 days. The

retrieved membranes were washed with deionized water, dried in vaccum oven weighed until constant weight is attained.

DETERMINATION OF TOXICITY

Cell culture

For biological evaluation, mouse fibroblast L929 cells were procured from the National Centre for Cell Science, Pune, India. The cells were grown in DMEM supplemented with 10% FBS and containing the antibiotics penicillin, streptomycin and amphotericin B (5000 units) in a humidified incubator at 5%CO_2 at 37 ± 0.2^{0}C. The cells were regularly monitored by phase contrast inverted light microscopy. The medium was changed once in three days. The confluent monolayer was sub-cultured and maintained for further studies

Sample preparation

Samples were exposed in Ultraviolet irradiation (Biogenix Research Center, Trivandrum, India) for 30 minutes and was directly taken for the analysis.

Evaluation of the toxicity of material extracts by MTT assay

The cytotoxicity of material was evaluated as per ISO10993-5 on L-929 mouse fibroblast cell culture. The cells were seeded onto a 48 well plate and incubated. After attaining confluency, the sterile material was added to the cell seeded plate. The percentage of the surviving fibroblast cells were quantified by the MTT assay and the morphological changes of the cells were monitored by phase contrast microscopy.

MTT assay is carried out to measure mitochondrial cellular metabolism (viability) and number of viable cells. MTT assay is based on the capability of

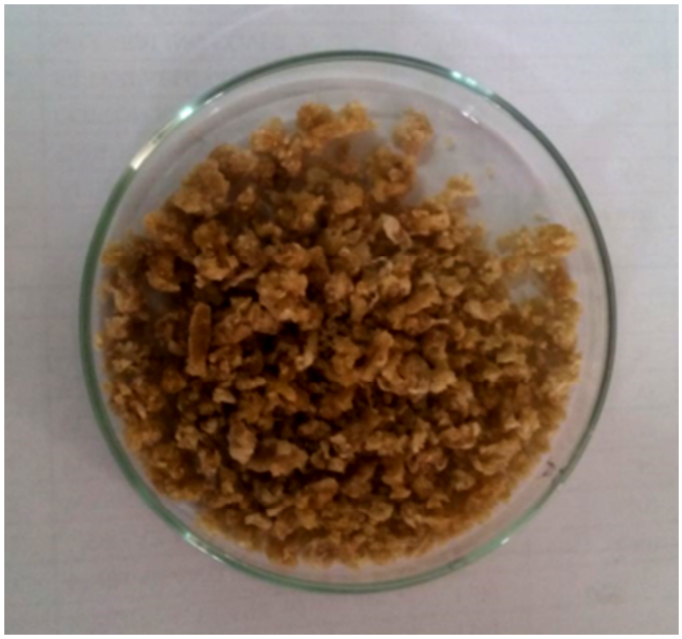
metabolically active fibroblast cells to reduce the yellow water-soluble tetrazolium salt (MTT) to purple formazan crystals using the mitochondrial enzyme succinate dehydrogenase (SDH). The intensity of purple colour so formed is proportional to the number of viable cells. Following the experiment the culture was washed with 1 x PBS and then 200 µl MTT solution per ml culture (MTT 5 mg/ml dissolved in PBS and filtered through a 0.2 µm filter before use) were added. The whole content was again incubated at 37^{o}C for 3h and 300 µl DMSO were added to each culture well. The whole content was incubated at room temperature for 30 min until all cells were lysed and a homogenous colour was obtained. The solution was centrifuged for 2 min to sediment cell debris. The optical density (OD) was measured spectrophotometrically at 540 nm. Cells treated with MTT solution without sample was used as control. The percentage viability was calculated as follows.

$$\% \text{ Viability } = \frac{\text{OD of test}}{\text{OD of control}} \; X \; 100$$

Direct contact method

The cytotoxicity of materials under the direct contact of cell was determined by direct contact assay.L929 fibroblast cells(1x10^{4} cells/m) were seeded on to a well plate(3D Falcon) and allowed to proliferate to 24hrs to form a sub-confluent layer. Then the material (1cm diameter) was placed over the monolayer and allowed to proliferate for 24 hrs in a CO_2 incubator. After the incubation, cells were evaluated for changes in morphology with respect to control(cells grown without materials) under inverted phase contrast microscope(Olympus CKX41) attached with an imaging camera. The images were captured using imaging software Optika vision-pro.

Color Plates

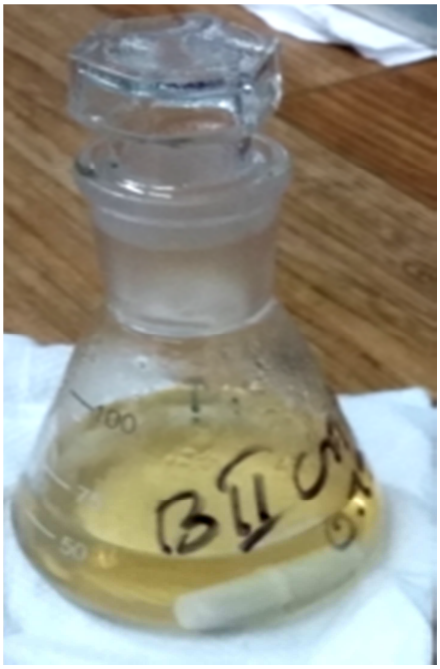


Chitosan[CP-1]

Strontium apatite[CP-2]

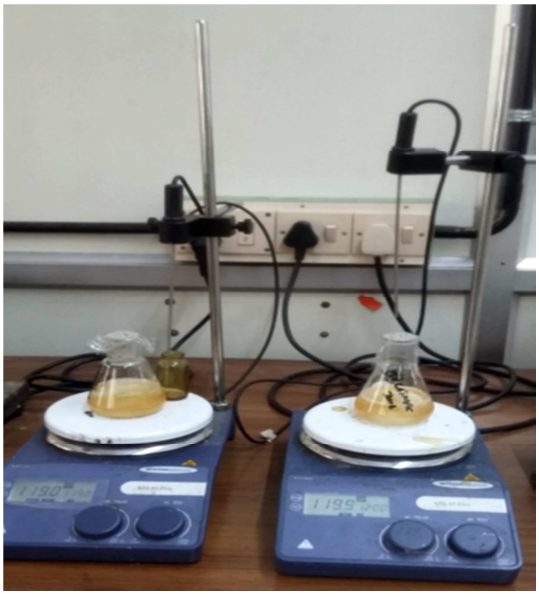
Acetic acid[CP-3]

Methanol[CP-4]



Conical flask containing magnetic bead[CP-5]

Dialysis membrane[CP-6]



Automatic stirrer[CP-7]

Electronic weighing machine[CP-8]

Freeze dryer[CP-9]

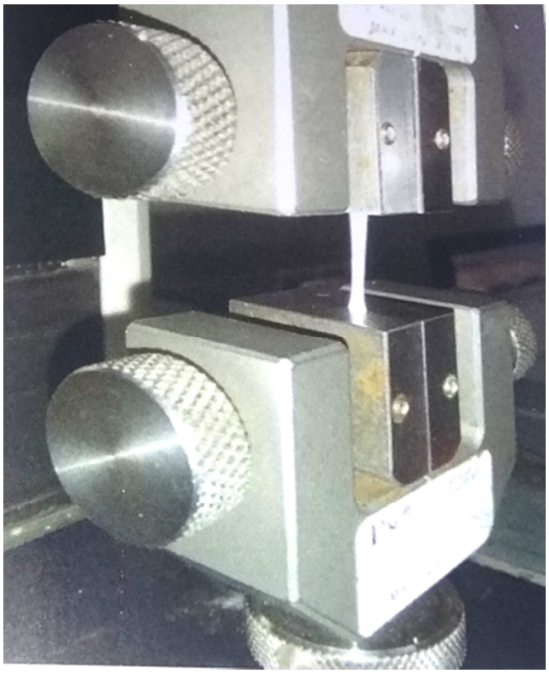
Scanning electron microscope[CP-10]

Thickness Guage[CP-11]

Universal testing machine[CP-12]

FTIR Spectrometer[CP-13]

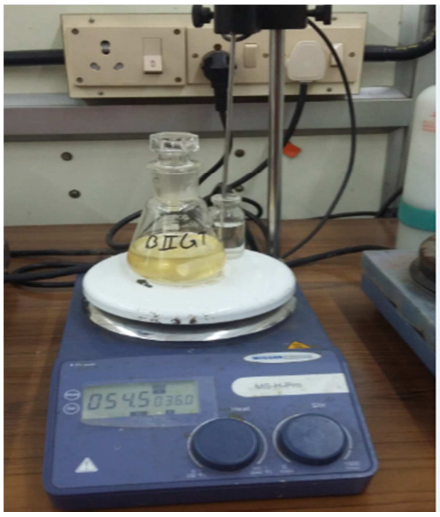
Phase Contrast Microscope[CP-14]

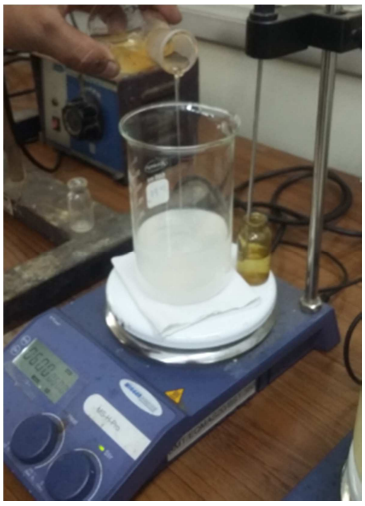


Measuring tensile strength[CP-17]

Measuring tear strength[cp[CP-18]

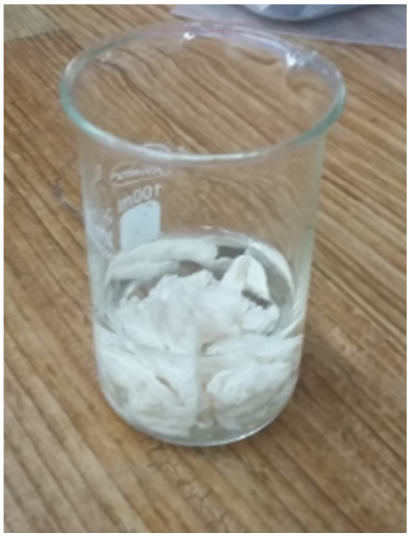
Preparation Of Chitosan Derivative[Cp-15]

(a) 

(b) 

(c) 

(d) 

(e) 

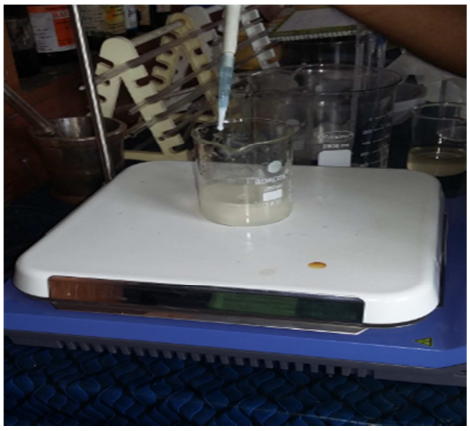
(f) 

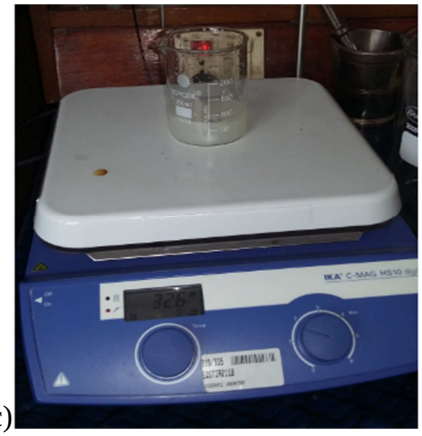
(g) 

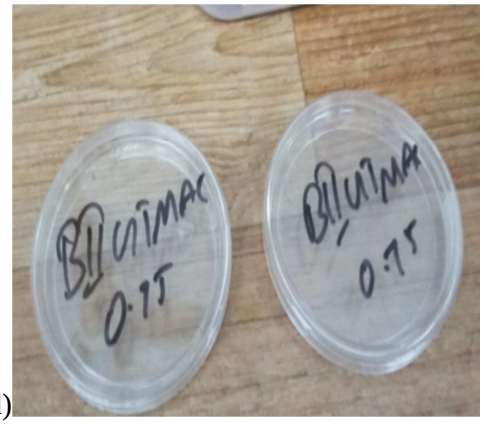
Preparation Of The Chitosan Derivative/Strontium Apatite

Composite Membrane[Cp-16]

(a) 

(b) 

(c) 

(d) 

Results & Observations

STATISTICAL ANALYSIS

The data is expressed in mean and standard deviation. Statistical package for social sciences(16.0)version used for analysis. ANOVA, POST HOC followed by dunnet t test applied to find the statistical significant between the groups. p value less than 0.05($p<0.05$)considered statistically significant at 95% confidence interval.

Group I-Chitosan derivative

Group II -Chitosan-Strontium Apatite Composite (7.5 mg)

Group III-Chitosan-Strontium Apatite Composite (10 mg)

1. MORPHOLOGICAL ANALYSIS

The morphology of the composite scaffold was examined with SEM. Image1a and 1b shows SEM images of Chitosan derivative under low magnification(200x) and high magnification (400x).It was showed high porosity and good inter pore connectivity with macropores of around 300µm for 400x and 500 µm for 200x and a lot of micropores. Image 2a,and 2b shows SEM images of chitosan derivative/strontium apatite composite membrane (7.5 mg) under low magnification(200x) and high magnification (400x).It was observed pore sizes of around 500µm and 300 µm. A good distribution of strontium apatite on the surface of scaffold s is also observed. Image 3a and 3b shows SEM images of chitosan derivative/strontium apatite composite membrane (10mg) under low magnification(200x) and high magnification (400x). Scaffold showed pore sizes 300µm and 500 µm. A good distribution of strontium apatite on the surface of scaffolds is also observed.

SEM IMAGES OF FABRICATED GTR MEMBRANES WITH DIFFERENT MAGNIFICATIONS

CHITOSAN DERIVATIVE

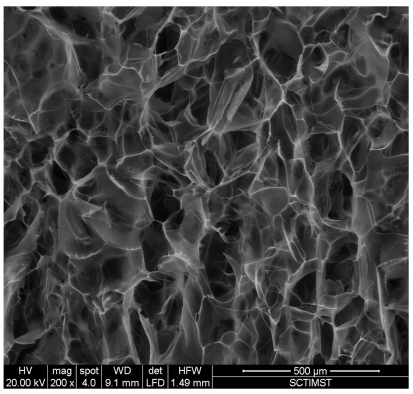


Image 1a

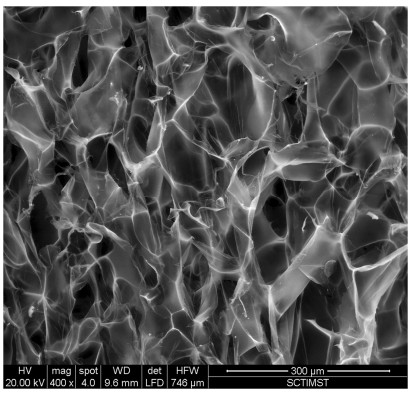


Image 1b

CHITOSAN-STRONTIUM APATITE COMPOSITE(7.5 MG)

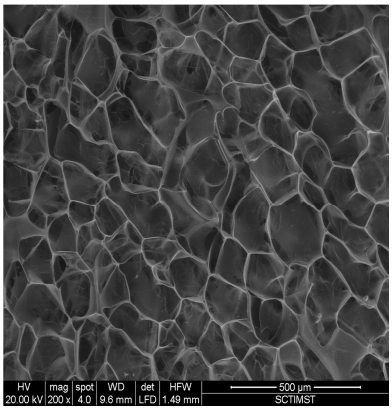


Image 2a

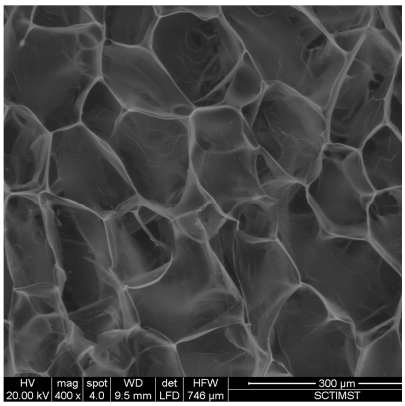


Image 2b

CHITOSAN-STRONTIUM APATITE COMPOSITE(10 MG)

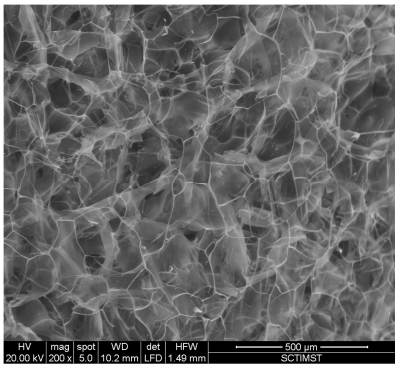


Image 3a

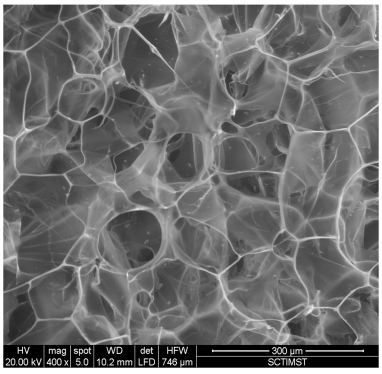


Image 3b

2. MEMBRANE THICKNESS

Table-1: Comparison of mean membrane thickness(mm) values between the groups

Groups	Treatment	Thickness (mm) (MEAN±SD)
Group-I	Chitosan derivative	0.25±0.01
Group-II	Chitosan-strontium apatite Composite (7.5 mg)	0.49±0.02*
Group-III	Chitosan-strontium apatite Composite(10 mg)	0.50±0.02*

(*p<0.05 significant compared Group-I with other groups, p>0.05 no significant difference compared Group-II with Group-III)

Table 1 shows Comparison of mean thickness values between the groups. Among this group I showed least thickness 0.25±0.01 when compared with group II [0.49±0.02] and group III [0.50±0.02] respectively and was not statistically significant compared with other groups. Group III shows highest thickness [0.50±0.02] and was statistically significant with group II. Overall there was an increase in thickness with the increase in weight of concentration of strontium apatite particles in composite membrane.

3. MECHANICAL PROPERTIES

Table-2: Multiple comparison of mean tensile strength (MPa) and elongation at break (%) (mm/mm) values between the groups

Groups	Tensile strength (MPa) (MEAN±SD)	Elongation at break (%) (mm/mm) (MEAN±SD)
Group-I	2.91±0.52	0.07±0.02
Group-II	0.47±0.13*	0.11±0.03*
Group-III	0.55±0.17*	0.12±0.01*

(*$p<0.05$ significant compared Group-I with other groups, $p>0.05$ no significant difference compared Group-II with other groups)

Table 2 shows Comparison of mean tensile strength and elongation at break value of between the groups. Among this group I showed no statistical significant when compared with group I and group III. Where group II was compared there was no statistical significant seen with group I and was statistical significant with group III. When group III was compared no statistical significant seen with group I and was statistical significant with group II.

4. SUTURE PULLOUT STRENGTH

Table-3: Multiple comparison of mean suture pull out strength values between the groups

Groups	Treatment	Suture pull out strength (MPa) (MEAN±SD)
Group-I	Chitosan derivative	0.31±0.11
Group-II	Chitosan-strontium apatite Composite(7.5 mg)	0.65±0.05*
Group-III	Chitosan-strontium apatite Composite(10 mg)	0.60±0.20*

(*$p<0.05$ significant compared Group-I with other groups, $p>0.05$ no significant difference compared Group-II with Group-III)

Table 3 shows multiple comparison of mean tear strength values between the groups. Among this group I showed no statistical significant when compared with group I and group III. Where group II was compared there was no statistical significant seen with group I and was statistical significant with group III. When group III was compared no statistical significant seen with group I and was statistical significant with group II.

5. CHEMICAL CHARACTERISATION OF SCAFFOLDS

FTIR Analysis

FTIR spectra was observed at 4000 cm-1 to 500cm-1, and different OH stretching was observed as: OH-NH vibration of chitosan alone at 3348cm-1,chitosan derivative at 3351cm-1, chitosan with 7.5 mg strontium apatite at 3285 cm-1 and 10mg strontium apatite at 3294cm-1.CH vibration of CH2 and CH3: chitosan alone at 2919.9 cm-1,chitosan derivative at 2877.2cm-1, chitosan with 7.5 mg strontium apatite at 2877.4 cm-1 and 10mg strontium apatite at 2877.1 cm-1.C=O stretching: chitosan alone at 1626.4cm-1,chitosan derivative at 1630.1cm-1, chitosan with 7.5 mg strontium apatite at 1633.3 cm-1 and 10mg strontium apatite at 1639.9cm-1.C-O-C group : chitosan alone at 1149.7cm-1,chitosan derivative at 1151.2cm-1, chitosan with 7.5 mg strontium apatite at 1151.2 cm-1 and 10mg strontium apatite at 1150.7cm-1. FTIR results clearly indicate the strong bonding between the Chitosan and strontium apatite. The results of FTIR shows existence of carbonate group along with the peaks of other groups. This results suggested that strontium apatite might be formed in the composite scaffolds with some carbonate incorporation.

6. IN-VITRO DEGRADATION TEST

Table-4: Comparison of mean initial dry weight of degradation between the groups

Days	Initial dry weight of degradation (MEAN±SD)			p value
	Group-I	Group-II	Group-III	
1st day	9.67±2.08	7.67±1.15*	7.33±0.57*	0.04
5th day	8.67±0.58	8.67±0.57	9.00±1.00	0.98
9th day	10.67±0.57	13.33±2.08*	9.33±2.30#	0.04
13th day	10.67±1.15	13.00±2.00*	9.67±1.52#	0.04
17th day	12.33±2.88	13.00±1.00	11.33±1.15	0.12
21st day	11.10±1.00	10.33±1.15	11.00±2.64	0.66
26th day	11.67±1.15	9.67±2.89	10.33±4.16	0.56
29th day	8.33±1.52	9.33±0.58	11.67±2.88	0.67

(*$p<0.05$ significant compared Group-I with other groups on same time period, #$p<0.05$ significant compared Group-II with other groups at same time period)

Table -4 shows Comparison of mean initial dry weight between the groups of degradation in PBS. Among this 1st, 9th and 13th day was statistically significant, p value 0.04.No statistical difference was found between other groups at 5th, 17th, 21st, 26th and 29th day.

Table:5: Comparison of mean weight after degradation between the groups

Days	weight after degradation (MEAN±SD)			p value
	Group-I	Group-II	Group-III	
1st day	9.67±2.08	7.67±1.15*	7.33±0.57*	0.04
5th day	8.33±1.15	8.67±0.58	9.00±1.00	0.23
9th day	8.33±0.57	12.33±1.52*	8.00±1.73#	0.04
13th day	9.00±1.00	13.00±2.00	9.33±1.15	0.06
17th day	9.00±1.73	10.67±0.58	9.66±1.15	0.08
21st day	7.67±0.57	9.67±1.15	9.67±1.52	0.32
26th day	8.00±0.00	8.00±1.73	8.33±2.51	0.56
29th day	6.00±1.73	8.33±0.57*	2.67±4.61*,#	0.03

(*$p<0.05$ significant compared Group-I with other groups on same time period, #$p<0.05$ significant compared Group-II with other groups at same time period)

Table -5 shows Comparison of mean dry weight between the groups after degradation in PBS. Among this 1st ,9th and 29th day was statistically significant , $p<0.05$.No statistical difference was found between other groups at 5th ,13th ,17th, 21st and 26th day.

Table-6: Comparison of mean degradation between the groups

Days	mean degradation (MEAN±SD)			p value
	Group-I	Group-II	Group-III	
1st day	0.00±0.00	0.00±0.00	0.00±0.00	-
5th day	0.41±0.07	0.00±0.00	0.00±0.00	0.56
9th day	0.21±0.04	0.06±0.07*	0.13±0.02*,#	0.03
13th day	0.15±0.05	0.00±0.00*	0.03±0.05*,#	0.03
17th day	0.26±0.03	0.17±0.08*	0.14±0.09*,#	0.04
21st day	0.29±0.08	0.06±0.10*	0.10±0.09*,#	0.01
26th day	0.31±0.06	0.16±0.0*	0.17±0.73*,#	0.02
29th day	0.28±0.08	0.10±0.06*	0.73±0.46*,#	0.03

(*$p<0.05$ significant compared Group-I with other groups on same time period, #$p<0.05$ significant compared Group-II with other groups at same time period)

Table 6 shows Comparison of mean degradation between the groups. Among this 9th,13th 17th 21st 26th and 29th was statistically significant , $p<0.05$.No statistical difference was found between 1st and 5th day between the groups. Overall there was an increase in weight loss with the increase in weight of concentration of strontium apatite particles in composite membrane.

7. IN-VITRO BIOACTIVITY TEST

Table-7: Multiple comparison of mean invitro bioactivity between the groups before and after 3 days in SBF.

Groups	Initial dry weight (mg) (MEAN±SD)	Final dry weight (mg) (MEAN±SD)	Weight difference (mg) (MEAN±SD)
Group-I	6.67±1.15	9.00±1.00	2.33±0.57
Group-II	8.00±1.73*	10.66±0.57*	2.66±1.15
Group-III	7.67±0.57*	11.00±2.73*	3.00±2.64*

(*p<0.05 significant compared Group-I with other groups, p>0.05 no significant compared Group-II with other groups)

Table 7 shows multiple comparison of mean invitro bioactivity between the groups before and after 3 days in SBF. Among this group I showed least weight loss [2.33±0.57] when compared with other groups which was statistically significant. Group III showed highest weight loss [3.00±2.64] which was statistically significant compared to group I and no statistical difference was seen when compared to group II. There is no statistical difference showed for group II when compared with group I and group III.

Table-8: Multiple comparison of mean invitro bioactivity between the groups before and after 7days

Groups	Initial dry weight (mg) (MEAN±SD)	Final dry weight (mg) (MEAN±SD)	Weight difference (mg) (MEAN±SD)
Group-I	5.33±0.57	7.67±0.57	2.33±0.57
Group-II	8.33±1.15*	12.00±1.00*	3.66±0.57*
Group-III	7.33±1.14*,#	11.00±1.00*,#	3.33±1.15*

(*$p<0.05$ significant compared Group-I with other groups, #$p<0.05$ significant compared Group-II with other groups)

Table 8 shows multiple comparison of mean invitro bioactivity between the groups before and after 7 days in SBF. Among this group I showed least weight loss [2.33±0.57] when compared with other groups which was not statistically significant. Group II showed highest weight loss [3.66±0.57] which was statistically significant compared with group I and no statistical difference compared to group II.

8. DETERMINATION OF TOXICITY

Table-9: Mean cell viability of the groups

Groups	Treatment	Cell viability (%) (MEAN±SD)
Group-I	Chitosan derivative	71.41
Group-II	Chitosan-strontium apatite Composite (7.5 mg)	85.69
Group-III	Chitosan-strontium apatite Composite (10 mg)	71.64

(a) chitosan derivative

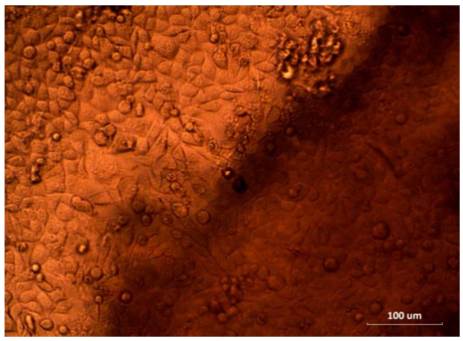
(b) chitosan-strontium apatite Composite (7.5 mg)

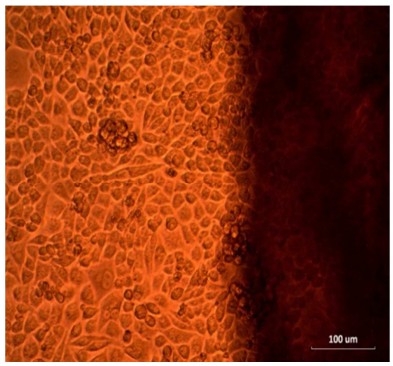
(c) chitosan-strontium apatite Composite (10 mg)**.**

Table shows Chitosan derivative membrane exhibited 71.41% cell viability. Among the composite membrane (chitosan derivative /strontium apatite 7.5mg) showed high percentage of viability(85.69%) after 24 hour. So on comparison composite membrane exhibited enhanced viability than chitosan derivative alone.

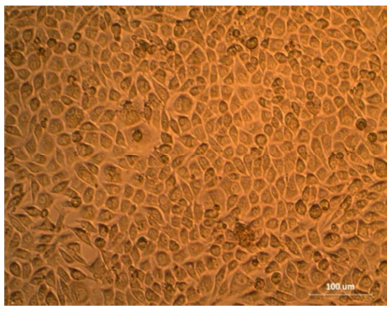
Direct contact assay with L-929 Mouse fibroblasts at 24 hours

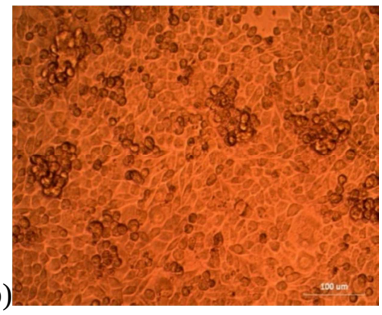
(a) 

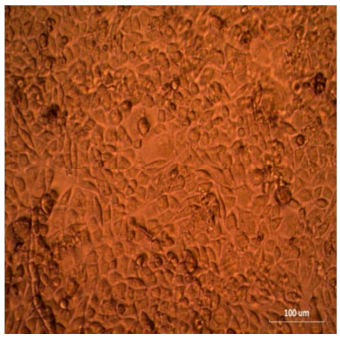
(b) 

(c) 

MTT assay with L-929 Mouse fibroblasts at 24 hours

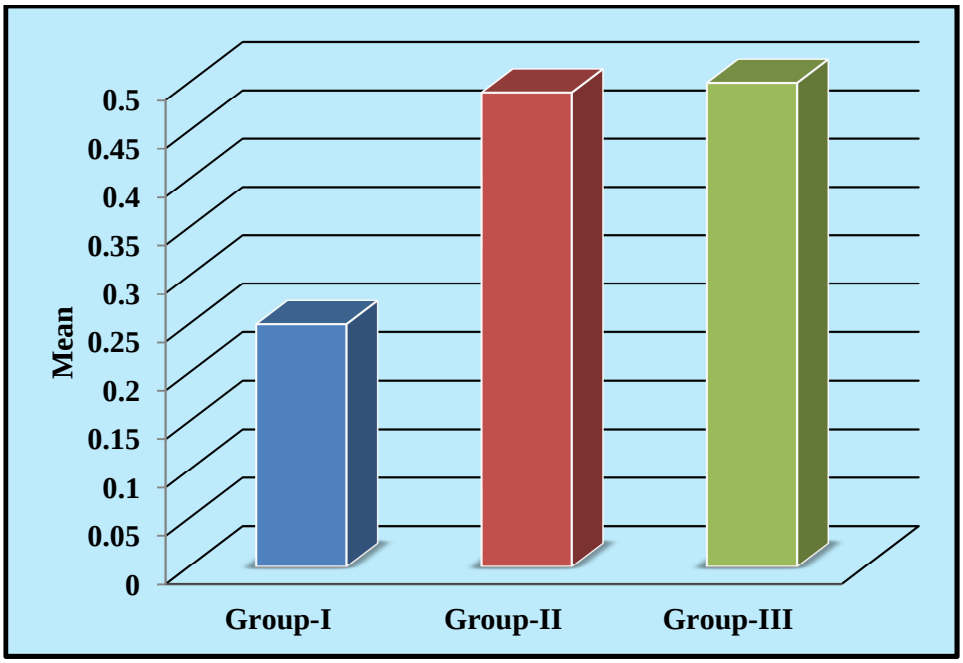
(a) 

(b) 

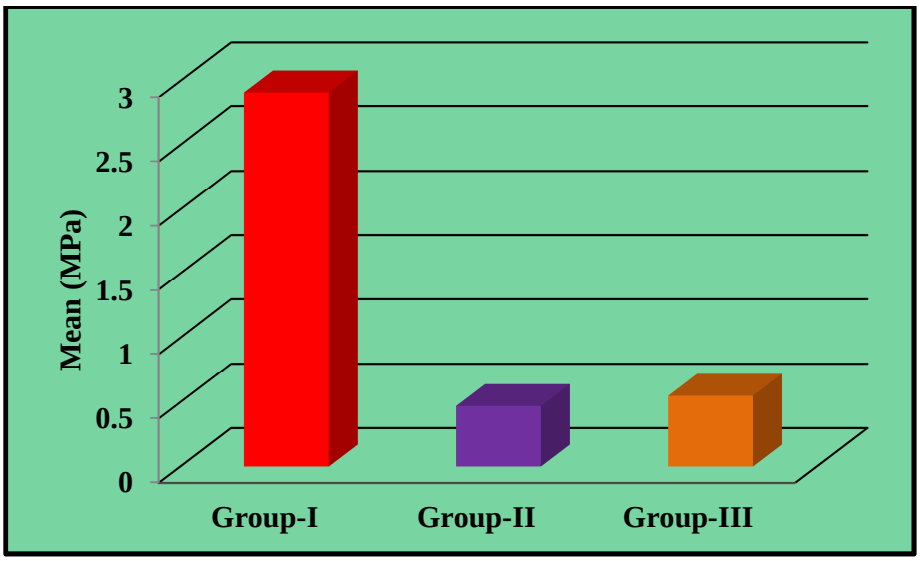
(c) 

Graphs

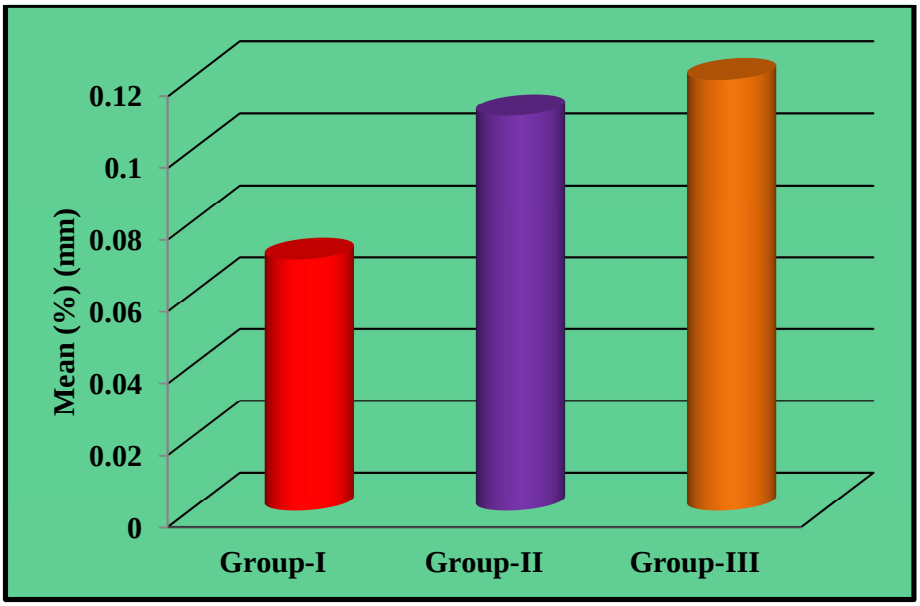
Graph-1: Comparison of mean thickness values between the groups



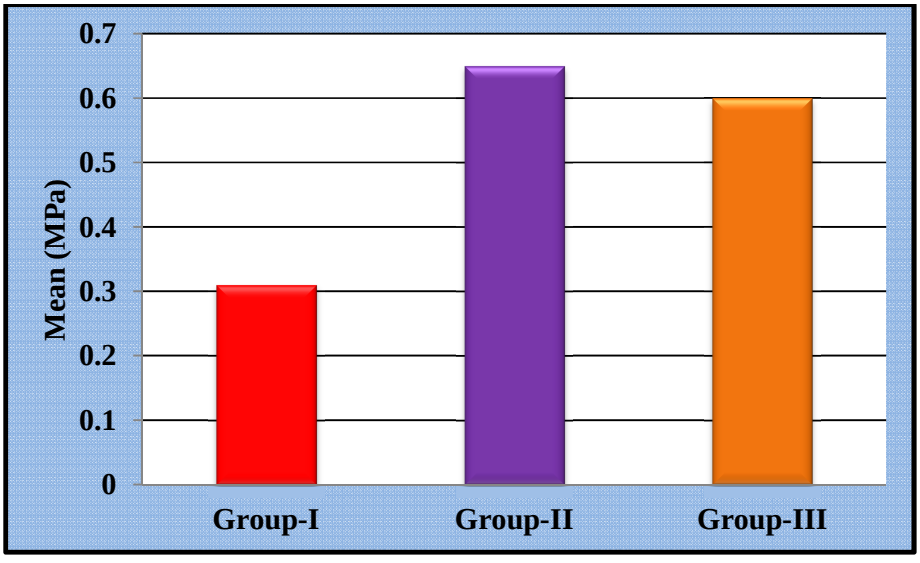
Graph-2: Multiple comparison of mean tensile strength (mg) values between the groups



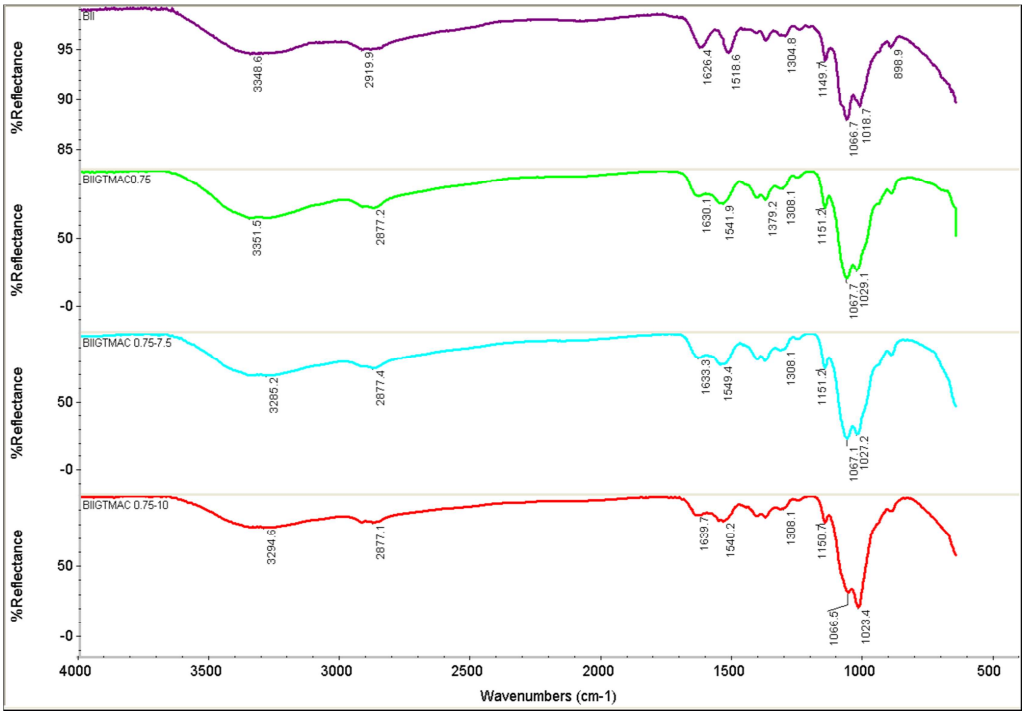
Graph-3: Multiple comparison of mean elongation at break (%)values between the groups



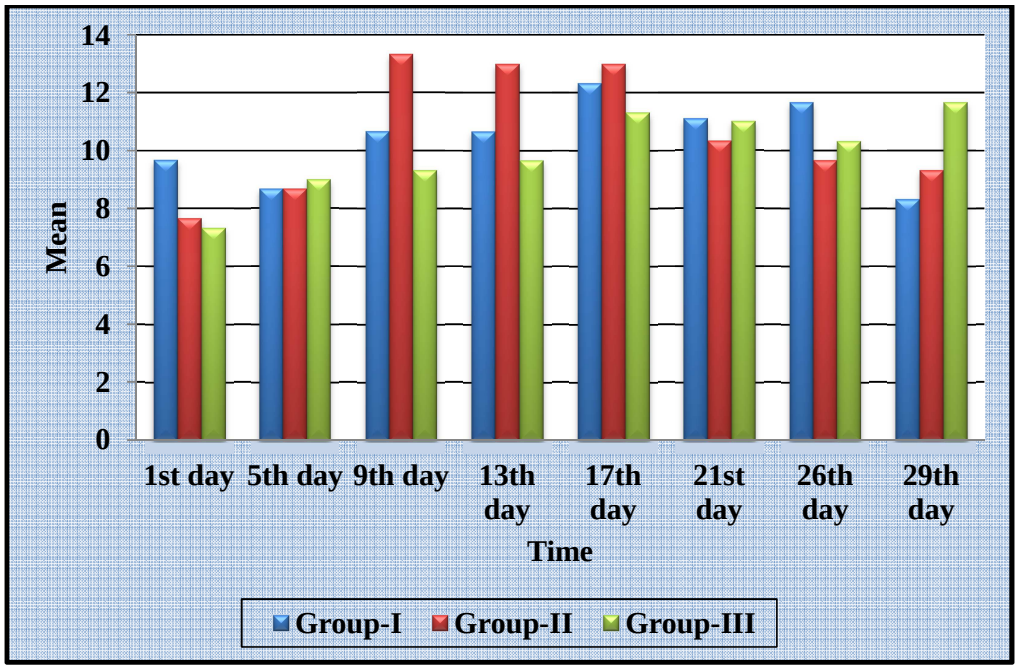
Graph-4: Multiple comparison of mean tear strength values between the groups



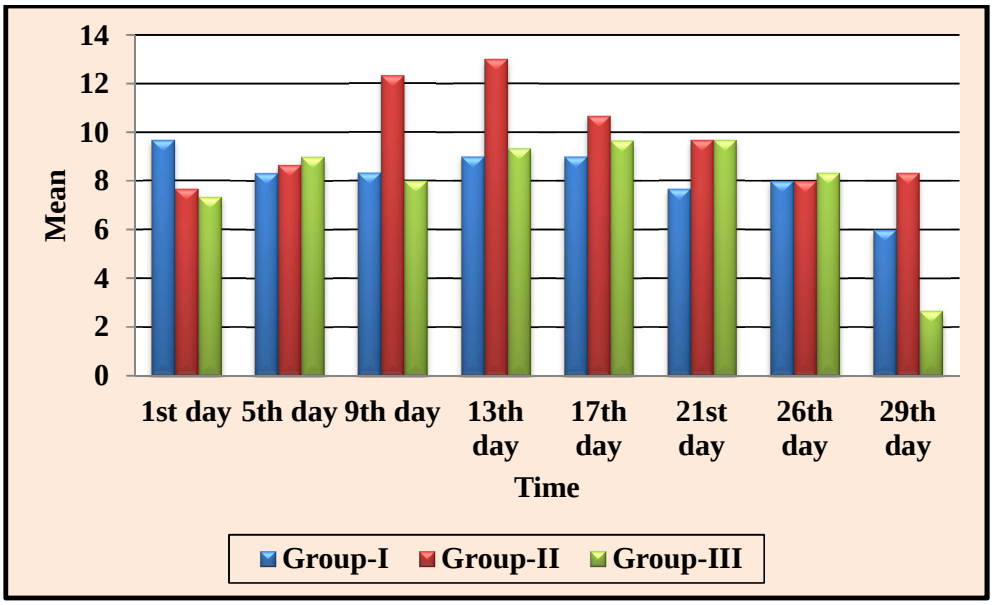
Graph-5: FTIR analysis between the groups



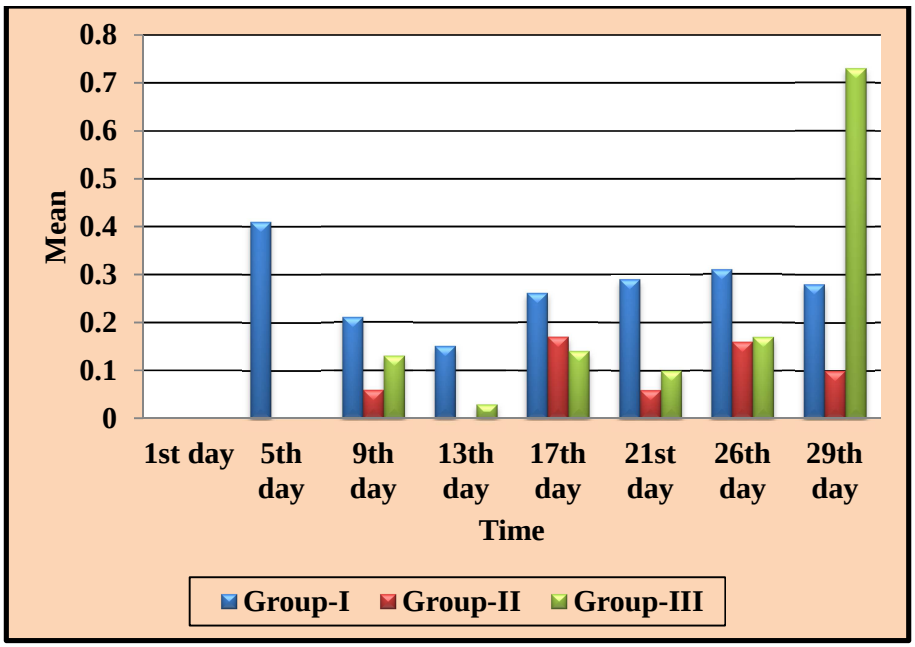
Graph-6: Comparison of mean initial dry weight of degradation between the groups



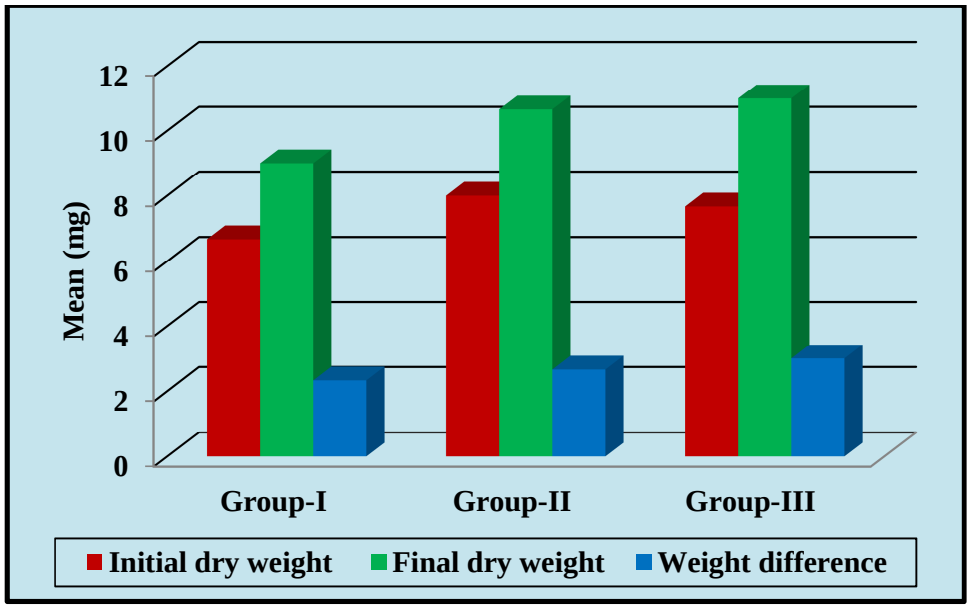
Graph-7: Comparison of mean weight after degradation between the groups



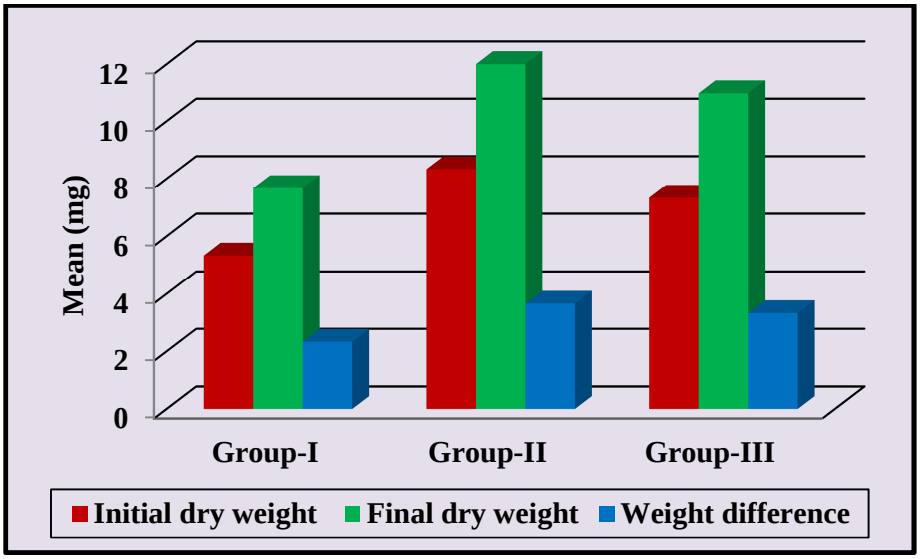
Graph-8: Comparison of mean degradation between the groups



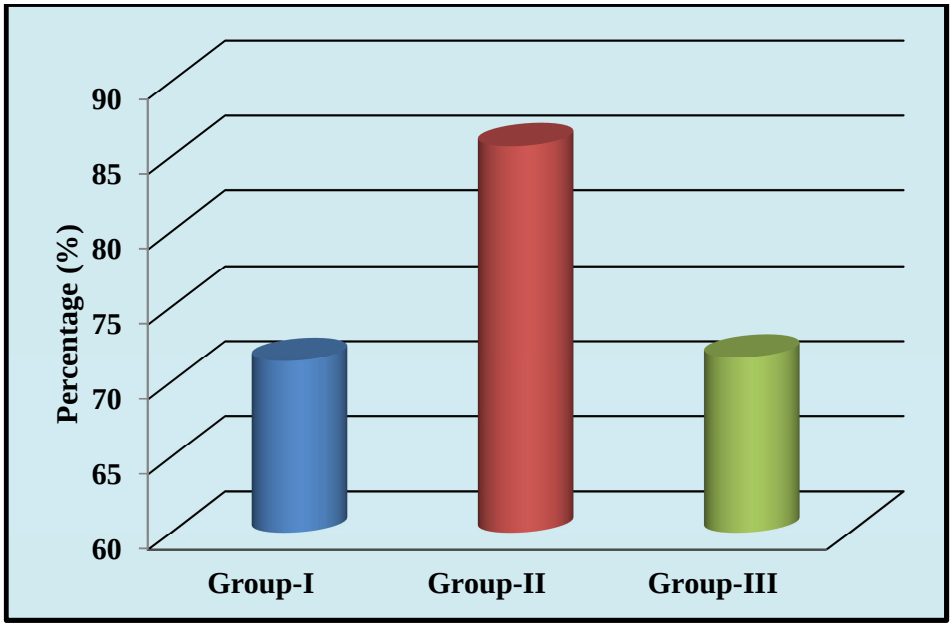
Graph-9: Multiple comparison of mean invitro bioactivity between the groups on 3 days in SBF.



Graph-10: Multiple comparison of mean invitro bioactivity between the groups on 7days in SBF.



Graph-11: Mean cell viability of the groups



Discussion

Periodontitis is an inflammatory disease of the supporting tissues of teeth caused by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament (PDL) and alveolar bone with an increase in probing depth, recession, or both.[45] The different approaches of periodontal therapy are pointed toward exclusion of etiological features, modification of anatomical defects, prevention of spread and elimination of symptoms of disease and regeneration of periodontal tissues. The several regenerative surgical procedure have been tried for the regeneration of periodontal tissues.[46]

Guided tissue regeneration is fundamentally the use of an occlusive membrane interfacing with the gingival connective tissue and epithelium on one side and periodontal ligament and alveolar bone tissues on the other side. It preserves space for clot stabilization and to stimulate periodontal tissue regeneration. It is created on the principle of exclusion of gingival connective tissue cells from the wound and preclusion of epithelial down growth. This method allows cells with regenerative potential to invade the wound site.[47] Progenitor cells situated in the remaining PDL, adjacent alveolar bone, or blood are then accomplished to recolonize the root area and differentiate with the development of new bone, PDL, and cementum.[48] Recent systematic reviews have verified the clinical advantages of guided regeneration procedures as opposed to conventional open-flap debridement for treating furcation and intrabony defects.[49] Such regenerative treatments include the utilization of a wide variety of surgical approaches, exogenous growth factors, barrier membranes, a series of bone grafts and osteoconductive materials or protein mixtures, genes from recombinant technology and cell-based technology.

Numerous barrier membranes have been established and proved as part of these two procedures to prevent epithelial and connective tissue cells from entering the deficient space, while allowing PDL cells to selectively migrate into the defect.

Barrier membranes should fulfil some important necessities:[50]

- Biocompatibility - the associations between membranes and host tissue should not induce adverse effect.
- Space-making - the ability to retain a space for cells from surrounding bone tissue to migrate for stable time duration.
- Cell- occlusiveness - inhibition of fibrous tissue that delay bone formation from entering the defect site.
- Mechanical strength - suitable physical properties to allow and keep the healing process, including protection of the underlying blood clot.
- Degradability - suitable degradation time matching the regeneration rate of bone tissue to avoid a secondary surgical procedure to eliminate the membrane.

First and second generation membranes act as a physical barrier to elude connective and epithelial tissue down growth into the defect for supporting the periodontal tissue regeneration. These barriers possess many mechanical, structural and bio functional restrictions. Due to the inherent limitations of nonresorbable barrier membranes, current research has been focused on the advance of resorbable membranes proper for clinical applications. The main benefit of resorbable membranes is avoiding a second surgical performance, which is required when non resorbable membranes are employed. Both natural and synthetic polymers are used to construct bioresorbable barrier membranes. Aquirre et al,[51] showed new bone

formation in bony defects by using absorbable membrane. However, controlling the time of absorption is difficult and later could cause a localized inflammatory response. In addition to the above disadvantages, poor membrane stability in the wet state causes space loss among the tooth and the membrane, creating reduced clinical results. The theory of tissue engineering has established, third-generation membranes have developed, which not only act as barriers but also as delivery devices to release specific agents such as antibiotics, adhesion factors, growth factors, etc., at the wound site on a time or need basis in order to coordinate and direct natural wound healing in a enhanced way.

Recently, attention in chitosan has improved due to its admirable biological properties such as biocompatibility, antibacterial effect, and rapid healing capacity. Some studies advocated that chitosan improves the formation of bone tissue and it could be used as the matrix of tissue engineering for gingiva. Paik et al,[52] reported that chitosan improved type I collagen synthesis in the early stage, and enabled differentiation into osteogenic cells in the human periodontal ligament fibroblasts in-vitro. In addition, a chitosan/collagen sponge applied to one wall intrabony defects surgically created in beagle dogs inhibited the apical migration of the epithelium and superior the growth of new bone and new cementum.[53]

Another biomaterial of attention is hydroxyapatite, which is a major component of human bone. Hydroxyapatite is used as bone substitute in the fields of orthopaedics and dentistry because of its good bioactivity, osteoconductivity, and biocompatibility. It is brittle and easy to fracture so it is difficult to mould into a specific shape. In order to overcome the drawback of hydroxyapatite, in this study we use strontiumapatite ie, strontium is incorporated into hydroxyapatite. It is

chemically similar to mineral component of bones, can form a direct chemical bond with surrounding bone tissues, ,inhibition of osteoclast formation, biodegradable, bioactive, osteoblast differentiation and excellent healing. Studies have revealed that nano-HA/chitosan composite scaffolds may serve as a good 3D substrate for cell attachment in vitro and migration in engineered bone and periodontal tissue. Complexes with natural polymer like chitosan and its derivatives with bioactive ceramic as a hydroxyapatite contribute and increase the appropriateness and advances in the field of TE. Such composite materials based on biodegradable polymers and bioactive ceramics, are appropriate for regenerative medicine.

In this study present study we fabricated three different GTR membrane, grouped as chitosan derivative, chitosan-strontium apatite composite (7.5 mg) and chitosan-strontium apatite composite(10 mg). Morphological analysis of fabricated membranes was done under SEM. Porous 3D scaffolds are commonly used in tissue engineering applications. The structural properties of the scaffolds, for example, porosity and pore size have direct implications on their functionality both in vitro and in vivo. Generally, interconnected porous scaffold networks that enable the transport of nutrients, removal of wastes, and facilitate proliferation and migration of cells are essential. Previous study by Murphy, C.M et al 2010 reported that optimal cell proliferation and infiltration was found in CG scaffolds with mean pore sizes greater than 300μm.[54] In addition, the ability of larger pores to aid cell infiltration was shown to override the beneficial effect of greater initial cell attachment surface areas provided by smaller pores. This study supported the importance of having pore sizes greater than 300μm for osteogenesis to occur. Artel et al 2011 showed that larger pore sizes of approximately 160 to 270μm enabled

angiogenesis throughout scaffold by using multi-layered agent-based model simulation.[55] It has also been revealed that vascularization of constructs necessitates pores greater than 300μm. Scaffolds with pore sizes of about 20 to 1500 μm have been used. In this study scaffolds produced from chitosan derivative concentration had better porous structures of pore sizes 300 μm and 500 μm under different magnification. With the incorporation of strontium apatite the porous structure of the composite scaffolds did not change significantly. Here chitosan derivative and chitosan derivative/strontium apatite composite scaffolds (7.5 mg and 10 mg strontium apatite) showed high porosity and good inter pore connectivity ie, 300 μm and 500 μm, serve to provide suitable microenvironments to support cell growth and function. Compared to the chitosan derivative the pore sizes of composite scaffolds decreased slightly. It was observed that good distribution and good adhesion of strontium apatite particles in the chitosan matrix were present.

The mechanical properties of the fabricated membranes observed in the study included thickness, tensile strength, elongation at break and suture pull out strength. All the membrane exhibited statistical significant difference in mechanical properties within the group. Among the Comparison of mean thickness within the groups, composite membranes exhibited increased thickness compared with chitosan derivative. Graph 1 shows Comparison of mean thickness values between the groups. Among this group I shows least thickness compared with group II and group III respectively and was statistically significant compared with other groups. Group III shows highest thickness and was not statistically significant with group II. Overall there was an increase in thickness with the increase in weight of concentration of strontium apatite particles in composite membrane. Among the

comparison of tensile strength within the groups, [graph 2] chitosan derivative exhibited highest tensile strength compared with composite membranes. The composite membrane showed a significant decrease in the tensile strength compared to chitosan derivative. In this study chitosan derivative /strontium apatite composite membrane have tensile strength 0.55±0.17MPa.The significant decrease in tensile strength for the composite membrane can be attributed to a great number of open pores. But it possess sufficient tensile strength to function as a barrier membrane. Previous study by Hunter KT et al. in 2013 reported hydroxyapatite–chitosan–gelatin membranes have comparable tensile strength (0.5–10MPa) and have better or comparable mechanical properties and possess sufficient tensile strength to function as a barrier membrane.[37]

Considering the elongation break or elasticity of fabricated GTR membrane,[Graph 3]highest elongation of break exhibited by chitosan –strontium apatite(7.5mg and 10mg) compared with chitosan derivative .Chitosan derivative [0.07±0.02] shows least elongation at break and chitosan –strontium apatite(10mg) shows highest[0.12±0.01] elongation at break. There was statistical difference between chitosan derivative with composite membranes. Overall there was an increase in elongation at break with the increase in weight of concentration of strontium apatite particles in composite membrane.

Previous study by P. A. Norowski et al.2012, evaluated Suture pull out strength and in vitro fibroblast and RAW 264.7 monocyte biocompatibility of genipin cross linked nano fibrous chitosan mats for guided tissue regeneration. He concluded that ultimate suture pull out strength was significantly lower (51–67%) than that of commercially available collagen membranes.[36] In this study, the tear

strength of the chitosan derivative membranes were less than that of composite membrane[graph 4] .Chitosan derivative showed least tear strength compared with other groups and chitosan-strontium apatite (7.5mg) showed highest tear strength. Overall there was an increase in tear strength with the increase in weight of concentration of strontium apatite particles in composite membrane.

Evaluating chemical stability of the fabricated membrane by FTIR, [Graph 5] the spectra revealed that OH-NH vibration of chitosan alone at 3348cm-1,chitosan derivative at 3351cm-1, chitosan with 7.5 mg strontium apatite at 3285 cm-1 and 10mg strontium apatite at 3294cm-1.CH vibration of CH2 and CH3: chitosan alone at 2919.9 cm-1,chitosan derivative at 2877.2cm-1, chitosan with 7.5 mg strontium apatite at 2877.4 cm-1 and 10mg strontium apatite at 2877.1 cm-1.C=O stretching : chitosan alone at 1626.4cm-1,chitosan derivative at 1630.1cm-1, chitosan with 7.5 mg strontium apatite at 1633.3 cm-1 and 10mg strontium apatite at 1639.9cm-1.C-O-C group : chitosan alone at 1149.7cm-1,chitosan derivative at 1151.2cm-1, chitosan with 7.5 mg strontium apatite at 1151.2 cm-1 and 10mg strontium apatite at 1150.7cm-1. FTIR results specify the strong bonding between the Chitosan and strontium apatite. The result of FTIR shows existence of carbonate group along with the peaks of other groups. Finding of the study suggested that strontium apatite might be formed in the composite scaffolds with some carbonate incorporation.

In degradation analysis the composites containing strontium apatite experienced higher weight loss. Graph 8 shows Comparison of mean degradation between the groups. Among this $9^{th,}$ 13^{th} 17^{th} 21^{st} 26^{th} and 29^{th} was statistically significant. No statistical difference was found between 5^{th} day between the groups.

Overall there was an rise in weight loss with the rise in weight of concentration of strontium apatite particles in composite membrane. This is associated to the favoured attack at the polymeric–ceramic interface resulting in leaching of strontium apatite to the solution. Finding from our study consistent with the study done by Tao Sun et al, 2014, similar results were reported for other biodegradable polymers reinforced with hydroxyapatite.[38] Invitro bioactivity within the groups between 3 and 7 days in SBF [Graph 13]. Chitosan derivative is least weight loss compared with other groups and there is no statistical difference showed between chitosan-strontium apatite composite membranes. Overall there was an rise in weight loss with the rise in weight of concentration of strontium apatite particles in composite membrane.

The most important requirement for the biomaterial is its biocompactibility in a specific environment, together with the non-cytotoxicity of its degradation products.as a preliminary step towards the evaluation of cyto- compactability of the scaffold, MTT assay was performed and result reveals the non-cytotoxicity nature of the tested membranes i.e, chitosan derivative, chitosan-strontium apatite Composite(7.5 mg) and chitosan-strontium apatite Composite(10 mg). The percentage of viability of cells on the membrane determine the suitability of the material for the intended application.in this study the potential of fabricated membranes for the tissue engineering applications was evaluated by invitro cell culture studies using L929 mouse fibroblast cell lines. The cells maintained their characteristics spindle morphology on all the GTR membranes after 24 hours. Chitosan derivative membrane exhibited 71.41% cell viability. Among the composite membrane (chitosan derivative /strontium apatite 7.5mg) showed high

percentage of viability(85.69%) after 24 hours [Graph 14]. So on comparison composite membrane exhibited enhanced viability than chitosan derivative alone.

One of the limitation of the study was in cell part. Present study analysed only the cytotoxicity. In the future the membrane should be completely tested for cell studies. ie, cell adhesion, proliferation, migration, differentiation.

This study highlights the outcome of cross-linking chitosan derivative and Strontium apatite and could be a potential approach to increase the morphological properties, mechanical strength and possess excellent degradation behaviour, in-vitro bioactivity finally enhanced cell viability. The challenges of TE are principally the physicochemical properties of the scaffold, surface chemistry and biological adaptation for cell culture. In line with these cross-linked chitosan derivative and strontium apatite scaffold are promising substrate for guided tissue regeneration membrane.

Summary

In the present study an attempt is being made to fabricate third generation GTR membrane for periodontal tissue engineering application.ie, chitosan derivative and strontium apatite of varying concentration via freeze drying technique and comparing their in vitro properties. All the fabricated scaffolds were highly porous and had interconnected pore structures. The addition of strontium apatite to chitosan derivative enhanced mechanical properties of composite scaffold compared to chitosan derivative. The composite membrane showed excellent degradation property and in-vitro bioactivity. There was an increase in weight loss with the increase in weight of concentration of strontium apatite particles in composite membrane. Evaluating chemical stability of the fabricated membrane by FTIR ,shows existence of carbonate group along with the peaks of other groups suggested that strontium apatite might be formed in the composite scaffolds with some carbonate incorporation. In cell culture ;direct contact and MTT assay, the scaffolds showed positive response to mouse fibroblast L929 cells attachment.

Conclusion

Biomaterials consist of bioactive and bioresorbable substances which mimic the natural function of bone and activate in vivo mechanisms of tissue regeneration. Such composite materials based on biodegradable polymers and bioactive ceramics, are suitable for regenerative medicine. In this present study chitosan derivative and chitosan derivative/strontiumapatite composite scaffolds were successfully fabricated and characterised. The morphological properties, mechanical properties, chemical properties, degradation behaviour and bioactivity of the scaffolds were studied. Moreover, the scaffolds showed positive response to mouse fibroblast L929 cells attachment. Overall, the findings suggest that strontium apatite -chitosan derivative composite scaffolds could be suitable for use as a GTR membrane. Further studies are needed for chitosan derivative and strontium apatite composite membrane for clinical use.

Bibliography

1. Albandar JM. Epidemiology and risk factors of periodontal diseases. Dent Clin North Am 2005;49:517.
2. Greenwell H. Position paper: Guidelines for periodontal therapy. J Periodontol 2001;72:1624-8.
3. Karring T, Nyman S, Gottlow J, Laurell L. Development of the biological concept of guided tissue regeneration – animal & human studies. Periodontol 2000 1993;1:26-35.
4. Sam G, Pillai BRM. Evolution of Barrier Membranes in Periodontal Regeneration-Are the third Generation Membranes really here?. J Clin Diag Res 2014;8(12):ZE14-ZE17.
5. Hench LL, Polak, JM. Third-generation biomedical materials. Science 2002;295:1014-7.
6. Pandit N, Malik R, Philips D. Tissue engineering in periodontics. J Indian Soc Periodontol 2011:15(4):328-37.
7. Bartold PM, Gronthos S, Ivanovski S, Fisher A, Hutmacher DW. Tissue engineered periodontal products. J Periodont Res 2016; 51:1-15.
8. Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues state of the art and future perspectives. J Biomater Sci Polym Ed 2001;12(1):107-24.
9. Shukla S. Freeze Drying Process: A Review. IJPSR 2011; Vol. 2(12): 3061-8.
10. Mizo S, Singh D, Kumar A, Cho YW, Oh TH, Han SS. Chitosan-hydroxyapatite macroporous matrix for bone tissue engineering. Curr Sci 2012;103(12):25.

11. Ahmed S, Ahmad M, Ikram S. Chitosan. A Natural Antimicrobial Agent- A Review. J Appl Chem 2014;3(2):493-503.
12. Weng L, Teusink MJ, Shuler FD, Parecki V, Xie J. Highly controlled coating of strontium-doped hydroxyapatite on electrospunpoly(E-caprolactone) fibers. J Biomed Mater Res Part B 2016:00B:000–000.
13. Venkatesan J, Kim S. Chitosan Composites for Bone Tissue Engineering - An Overview. Drugs 2010;8:2252-66.
14. Young-Ku et al. Chitosan/poly(L-lactic acid) multilayered membrane for guided tissue regeneration, J Biomed Mater Res 2016;90A:766-72.
15. Karring T, Lindhe J, Cortellini P. Regenerative periodontal therapy. In: Lindhe J, Karring T, Lang NP, editors. Clinical Periodontology and Implant Dentistry. Copenhagen: Blackwell Publishing Company; 2003. p 674.
16. Xu C, Lei C, Meng L, Wang C, Song Y. Chitosan as a barrier membrane material in periodontal tissue Regeneration. J Biomed Mater Res Part B Appl Biomater 2012;100(5):1435-43.
17. Chavanne P, Stevanovic S, Wüthrich A, Braissant O, Pieles U, Gruner P, Schumacher R. 3D printed chitosan / hydroxyapatite scaffolds for potential use in regenerative Medicine. Biomed Tech 2013; 58(1).
18. Pighinelli L, Kucharska M. Properties and Structure of Microcrystalline Chitosan and Hydroxyapatite Composites. J Biomater Nanobiotechnol 2014;5:128-38.
19. Kumirska J, Weinhold MX, Thoming J, Stepnowski P. Biomedical Activity of Chitin/Chitosan Based Materials - Influence of Physicochemical Properties Apart From Molecular Weight and Degree of N-Acetylation. Polymers 2011;*3*(4):1875-901.

20. Badawy MEI, Rabea E. A Biopolymer Chitosan and its Derivatives as promising antimicrobial agents against plant pathogens and their applications. In Crop Protection. Int J Carbohydrate Chem 2011;Article ID 460381, 29 pages

21. Ahmed S, Ikram S. Chitosan and its Derivatives: A Review in Recent Innovations. IJPSR 2015;6(1):14-30.

22. Zo SM, Deepti S, Ashok K, Cho YW, Oh TH, Han SS. Chitosan-Hydroxyapatite Macroporous Matrix for Bone Tissue Engineering. Curr Sci 2012;103(12):1438-46.

23. Yin YJ, Zhao F, Song XF, Yao KD, Lu WW, Leong C. Preparation and Characterization of Hydroxyapatite/Chitosan-Gelatin Network Composite. J Appl Poly Sci 2000;77:2929-38.

24. Zhao F, Yin Y, Lu WW, Leong JC, W Zhang, Zhang J, Zhang M, Yao K. Preparation and histological evaluation of biomimetic three dimensional HA/chitosan-gelatin network composite scaffolds. Biomaterials 2002;23(15): 3227-323.

25. Yamaguchi I, Iizuka S, Osaka A, Monma H, Tanaka J. The effect of citric acid addition on chitosan/hydroxyapatite composites. Colloids and Surfaces A: Physicochem Eng Aspects 2003;214:111-8.

26. Park JS, Choi SH, Moon IS, Cho KS, Chai JK, Kim CK. Eight-week histological analysis on the effect of chitosan on surgically created one-wall intrabony defects in beagle dogs. J Clin Periodontol 2003;30:443-53.

27. Chen TW, Kuo SM, Chang SJ, Kuan TC. Fabrication and evaluation of chitosan membranes for guided tissue regeneration. Biomed Eng Appl Basis Comm 2004;16:259-264.

28. Im KH, Park JH, Kim KN, Kim KM, Choi SH, Kim CK, Lee YK. Organic-Inorganic Hybrids of Hydroxyapatite with Chitosan. Key Eng Mater 2005;284-286:729-32.

29. Kong L, Gao Y, Cao W, Gong Y, Zhao N, Zhang X. Preparation and characterization of nano-hydroxyapatite/chitosan composite scaffolds. J Biomed Mater Res A 2005;75(2):275-82.

30. Kuo SM, Chang SJ, Chen TW, Kua TC. Guided tissue regeneration for using a chitosan membrane: An experimental study in rats. Inc. J Biomed Mater Res 2006;76A:408-15.

31. Zainol I, Zakaria FA, Saliman MR, Derman MA. Preparation and characterisation of chitosan/nano hydroxyapatite composites. Solid State Sci Technol 2008;16(1):153-9.

32. Shin JA, Choi JY, Kim ST, Kim CK, Lee YK, Cho KS, et al. The effects of hydroxyapatite- chitosan membrane on bone regeneration in Rat Calvarial Defects. J Korean Acad Periodontol 2009;39:213-22.

33. Garg T, Chanana A, Joshi R. Preparation of chitosan scaffolds for tissue engineering using freeze drying technology. IOSR J Pharm 2012;2(1):72-3.

34. Le HR, Qu S, Mackay RE, Rothwell R. Fabrication and mechanical properties of chitosan composite membrane containing hydroxyapatite particles. J Adv Ceramics 2012;1(1):66-71.

35. Huang Y, Han SG, Ding QQ, Yan YJ, Pang XF. Preparation of chitosan/ strontium-substituted hydroxyapatite films on titanium and its FTIR characteristics. Guang Pu Xue Yu Guang Pu Fen Xi 2013;33(9):2379-82.

36. Norowski PA, Mishra S, Adatrow PC, Haggard WO, Bumgardner JD. Suture pullout strength and in vitro fibroblast and raw 264.7 monocyte biocompatibility of genipin cross linked nanofibrous chitosan mats for guided tissue regeneration. J Biomed Mater Res A 2012;100(11):2890-6.

37. Hunter KT, Ma T. Invitro evaluation of hydroxyapatite-chitosan-gelatin composite membrane in guided tissue regeneration. J Biomed Mater Res 2013;101A:1016-25.

38. Sun T, Khan TH, Sultana N. Fabrication and in vitro evaluation of nanosized Hydroxyapatite/Chitosan based tissue engineering scaffolds. J Nanomater 2014, 8 pages.

39. Pighinelli L, Kucharska M. Properties and structure of microcrystalline chitosan and hydroxyapatite composites. J Biomater Nanobiotechnol 2014;5:128-38.

40. Bavariya AJ, Andrew Norowski P, Mark Anderson K, Adatrow PC, Garcia-Godoy F, Stein SH, Bumgardner JD. Evaluation of biocompatibility and degradation of chitosan nanofiber membrane cross linked with genipin. J Biomed Mater Res Part B 2014;102B:1084-92.

41. Escobar-Sierra DM, Martins J, Ossa-Orozco CP. Chitosan/Hydroxyapatite Scaffolds for Tissue Engineering Manufacturing Method Effect Comparison, Rev Fac Ing Univ Antioquia N.º75 2015:24-35.

42. Sahai N, Tewari RP. Characterization of effective mechanical strength of chitosan porous tissue scaffolds using computer aided tissue engineering. Int J Biomed Eng Sci 2015;2(1):21-8.

43. Tu Y, Chen C, Li Y, Hou Y, Huang M, Zhang L. Fabrication of nano-hydroxyapatite/chitosan membrane with asymmetric structure and its applications in guided bone regeneration. Biomed Mater Eng 2017;28(3):223-33.

44. Park SM, Kim HS. Preparation of acetylated chitosan/carbonated hydroxyapatite composite barriers for guided bone regeneration. Macromolecular Research 2017;25(2):158-64

45. Newman MG, Takei HH, Carranza NT. Carranza's Clinical Periodontology, 11th ed., 2012. Saunders Elsevier, St-Louis, MO, USA.

46. Sculean A, Nikolidakis D, Schwarz F. Regeneration of periodontal tissues: combinations of barrier membranes and grafting materials-biological foundation and preclinical evidence: a systematic review. J Clin Periodontol 2008;35:106-23.

47. Wang HL, Greenwell H, Fiorellini J, Giannobile W, Offenbacher S, et al. Periodontal regeneration. J Periodontol 2005;76(9):1601-22.

48. Scannapieco FA. Periodontal regeneration techniques for treatment of periodontal diseases. Dent Clin N Am 2005;49:637-59.

49. Murphy KG, Gunsolley JC. Guided tissue regeneration for the treatment of periodontal intrabony and furcation defects. A systematic review. Ann Periodontol 2003;8:266-81.

50. Zhang Y, Zhang X, Shi B, Miron RJ. Membranes for guided tissue and bone regeneration. Annals of Oral & Maxillofacial Surgery 2013;1(1):10.

51. Aquirre-Zorzano LA, Estefania-Cundin E, GilLozano J. Periodontal regeneration of infrabony defects using resorbable membrane: determinant of the healing

response. An observational clinical study. Int J Periodontics Rest Dent 1999;19:363-371.

52. Paik JW, Lee HJ, Yoo YJ. The effects of chitosan on the human periodontal ligament fibroblasts in vitro. J Korean Acad Periodontol 2001;31:823-832.

53. Park JS, Choi SH, Moon IS. Eight-week histological analysis on the effect of chitosan on surgically created one-wall intrabony defects in beagle dogs. J Clin Periodontol 2003;30:443-453.

54. Murphy CM, O'Brien FJ. Understanding the effect of mean pore size on cell activity in collagen-glycosaminoglycan scaffolds. Cell Adhesion & Migration. 2010;4(3):377-381.

55. Artel A, Mehdizadeh H, Chiu YC, Brey EM, Cinar A. An agent-based model for the investigation of neovascularization within porous scaffolds. Tissue Eng Part A 2011;17(17-18):2133-41.

Annexure

Annexure 1

Tele : 91 471 2340801 Fax : 91 471 2341814

श्री चित्रा तिरुनाल आयुर्विज्ञान तथा प्रौद्योगिकी संस्थान
बायो मेडिकल टैक्नोलॉजी विंग, पूजापुरा, तिरुवनन्तपुरम-695 012, इन्डिया

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
BIO MEDICAL TECHNOLOGY WING POOJAPPURA, THIRUVANANTHAPURAM-695 012, INDIA
(An Institute of National Importance under Govt. of India)

Date : 18 July 2016

Dr.Manoj Komath, Ph D
Scientist F, Bioceramics Division

To

Dr.Elizabeth Koshi
Principal
Sree Mookambika Institute of Dental Sciences
Kulashekharam

Sub : Permission for PG students to do research studies.

Sir/Madam,

As per your request dated 12.07.2016, I hereby promise to allow your PG student Dr. Shamna N.S. (Dept of Periodontics and Implantology) to conduct experiments in our lab, which are essential for her research work entitled "Fabrication and In vitro Characterization of Chitosan Derivative and Strontium Apatite Composite Sheets for Periodontal Tissue Engineering".

This will be done as an academic interaction, on collaborative terms and conditions between the Institutions.

Thanking you,

Sincerely,

(Manoj Komath)

Annexure 2

SREE MOOKAMBIKA INSTITUTE OF DENTAL SCIENCES

KULASEKHARAM, KANYAKUMARI DIST., TAMIL NADU, INDIA.

INSTITUTIONAL RESEARCH COMMITTEE

Certificate

This is to certify that the research project protocol, ***Ref no. 11/07/2016*** titled, ***"Fabrication and in-vitro characterization of chitosan derivative and strontium apatite composite sheets for periodontal tissue engineering"*** submitted by ***Dr. Shamna N. S., II Year MDS, Department of Periodontics*** has been approved by the Institutional Research Committee at its meeting held on ***26th July 2016.***

Convener
Dr. T. Sreelal

Secretary
Dr. Pradeesh Sathyan

Annexure 3

INSTITUTIONAL HUMAN ETHICS COMMITTEE

SREE MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES,
KULASEKHARAM, TAMILNADU

Communication of Decision of the Institutional Human Ethics Committee(IHEC)

SMIMS/IHEC No:1 /Protocol no:13 / 2016

Protocol title: Fabrication and in-vitro characterization of chitosan derivative and strontium apatite composite sheets for periodontal tissue engineering
Principal Investigator: Dr. Shamna N.S
Name& Address of Institution: Department of Periodontics and Oral Implantology Sree Mookambika Institute of Medical Sciences, Kulasekharam ☑ New review ☐ Revised review ☐ Expedited review
Date of review (D/M/Y): Date of previous review , if revised application:
Decision of the IHEC: ☑ Recommended ☐ Recommended with suggestions ☐ Revision ☐ Rejected
Suggestions/ Reasons/ Remarks:
Recommended for a period of :six months

Please note*

- Inform IHEC immediately in case of any Adverse events and Serious adverse events.
- Inform IHEC in case of any change of study procedure, site and investigator
- This permission is only for period mentioned above. Annual report to be submitted to IHEC.
- Members of IHEC have right to monitor the trial with prior intimation.

Reneegalgangadhar

Signature of Member Secretary IHEC

MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES
IHEC
Date 21.12.16
KULASEKHARAM